THE RELATIONSHIP BETWEEN BODY GROWTH, RETINAL BLOOD VESSEL DEVELOPMENT AND THE INCIDENCE OF RETINOPATHY OF PREMATURITY: A CLINICAL AND LABORATORY INVESTIGATION

BY

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ABSTRACT

BACKGROUND

Retinopathy of prematurity (ROP) is a vasoproliferative disorder of the retina in preterm infants and an important cause of childhood blindness. The incidence of ROP has varied over time and across the world as a result of changes in clinical neonatal practice. ROP is a multifactorial disease with low birth weight, low gestational age and exposure to supplemental oxygen being key risk factors. Prenatal and postnatal body growth together with availability of growth factors may also be involved in the pathogenesis. Premature infants undergo eye screening using binocular indirect ophthalmoscopy (BIO) or wide-field digital retinal imaging (WFDRI) in order to identify those infants with sight-threatening disease that require treatment with laser photocoagulation. Eye screening is a painful and distressing procedure for infants.

AIMS

(i) To report the trends in incidence of ROP within Lothian, Scotland
(ii) To report the incidence of ROP in small-for-gestational (SGA) infants
(iii) To study retinal vascular development in growth-restricted rat pups
(iv) To carry out a prospective randomised study comparing the diagnostic accuracy of WFDRI with BIO for ROP eye examinations
(v) To compare the pain experienced by infants undergoing WFDRI and BIO

METHODS

Data from eye screening examinations within Lothian between 1990 and 2004 were analysed and epidemiological data were obtained from the Scottish Health Service. Dams of rat pups were fed either a 'normal' (18%) or 'low' (9%) protein diet. Dams and pups were exposed to either room air or a fluctuating oxygen profile for 14 days. Retinas were dissected and blood vessels were stained using immunohistochemistry. The extent of vascular development was measured and compared between animal groups. Serum was collected from pups and levels of insulin-like growth factor-1 (IGF-1) were measured using an enzyme-linked immunosorbent assay. Infants from Edinburgh Neonatal Unit undergoing routine ROP screening were recruited and screened by both WFDRI and BIO in random order. The sensitivity and specificity of WFDRI compared to BIO was calculated. The pain experienced by infants was scored using the Premature Infant Pain Profile (PIPP) and compared between the two techniques.

RESULTS

There has been an increase in survival of preterm infants, a reduction in the incidence of any degree of ROP, severe ROP and a marked reduction in treatment for ROP in Lothian from 1990-2004. The prevalence of ROP and severe ROP were higher in SGA infants than their appropriately sized peers. Pups of dams fed the 9% protein diet were born growth restricted. The growth restricted rat pups had significantly larger retinal avascular areas and lower serum IGF-1 levels than 'normal' sized pups. The sensitivity of WFDRI in detecting any ROP, stage 3 ROP and plus disease was 60%, 57% and 80% respectively with a specificity of 91%, 98% and 98% respectively. ROP screening using WFDRI and BIO was painful but the pain experienced by infants was similar for both techniques (WFDRI PIPP score 15.0, BIO PIPP score 15.2).

CONCLUSIONS

Advances in obstetric and neonatal care within the developed world over the last two decades are likely responsible for the increased survival of premature infants and the overall decreasing incidence of ROP seen in Lothian. Body growth and IGF-1 are important in the pathogenesis of ROP. Further work is required to identify a mechanism for this association and clinical trials of nutritional therapies are needed. WFDRI is a useful examination technique for ROP eye screening but has some technical limitations which need to be improved. Further work is required to implement adequate analgesia regimes for ROP screening.

To Mum, Dad, Kev, Sachin, Amiya, Dhilan

CONTENTS

ABBREVIATIONS

AGA	appropriate-for-gestational age
ANOVA	analysis of variance
BIO	binocular indirect ophthalmoscopy
BOOST	Benefits of oxygen saturation targeting
BW	birth weight
CGA	corrected gestational age
CI	confidence interval
Cryo-ROP	cryotherapy for ROP
ELISA	enzyme-linked immunosorbent assay
ETROP	early treatment for ROP
GA	gestational age
HIF-1	hypoxia-inducible factor 1
HIF-1α	Hypoxia-Inducible Factor-1α
IGF-1	insulin-like growth factor 1
IGF-1R	IGF-1 receptor
IGFBP	IGF binding protein
IGFBP-3	insulin-like growth factor binding protein-3
IMR	infant mortality rate
ISD	Information services division
IUGR	intrauterine growth restriction
LB	live births
LREC	Lothian research ethics committee
MAPK	mitogen-activated protein kinases
NICU	neonatal intensive care unit
NIDCAP	Newborn individualised developmental care and assessment programme
PBS	phosphate-buffered saline
PFA	paraformaldehyde
PIPP	premature infant pain profile

ROP	retinopathy of prematurity
SD	standard deviation
SGA	small-for-gestational age
SMR	Scottish morbidity record
SUNDROP	Stanford university network for diagnosis of ROP
VEGF	vascular endothelial growth factor
VLBW	very low birth weight
WFDRI	wide-field digital retinal imaging
WINROP	Weight IGF-1 Neonatal ROP
X^2	Chi-square test
X^2 trend	Chi-square test for trend

DECLARATION

Except where due acknowledgement is made by reference, the studies undertaken in this thesis were the unaided work of the author. The work described in this thesis has not been previously accepted for, or is currently being submitted in candidature for another degree.

Chapters 2 and 3

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Chapter 4

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Chapters 5 and 6

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All chapters (2, 3, 4, 5, 6)

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CHAPTER 1

INTRODUCTION

Retinopathy of prematurity (ROP) is a vasoproliferative disorder of the retina in preterm infants. ROP can cause a range of visual deficits and is an important cause of childhood blindness worldwide.

1.1 Normal retinal blood vessel development (Figures 1.1-1.3)

There are two sources of blood supply to the retina:-the central retinal artery and the choroidal blood vessels. Both sources are derived from the ophthalmic artery. The choroidal blood vessels supply the uvea and outer and middle layers of the retina while the central retinal artery enters via the optic nerve head to nourish the inner retinal layer. Branches of the central retinal artery are first seen emerging from the centre of the optic disc when the fetus is approximately 16 weeks gestation. The blood vessels grow radially outwards towards the ora serrata and vascularisation is complete when the fetus is approximately 36 weeks gestation [Hughes *et al*, 2000].

Normal human retinal vascular development occurs as a result of vasculogenesis and angiogenesis [Hughes *et al*, 2000]. Vasculogenesis occurs first and involves the formation of blood vessels from endothelial precursor cells known as 'spindle' cells. Spindle cells are first seen emerging from the optic head and growing out towards the periphery across the superficial layer of the retina when the fetus is 14-15 weeks gestation. Spindle cells arrange to form cords which further develop into primitive vascular tubes. These tubes become associated with vascular smooth muscle cells and supporting pericytes [Hughes *et al*, 2000; Zhang and Stone, 1997]. Spindle cells do not reach the retinal periphery or the fovea and vasculogenesis is complete by the time the fetus is 21 weeks gestation.

Angiogenesis refers to the development of additional new blood vessels by budding from the existing primary vascular network. It is first seen when the fetus is 17-18 weeks gestation. Angiogenesis leads to the development of capillary networks within the existing vascular framework in the superficial layer of the retina. Angiogenesis also occurs from the leading vascular edge and forms the peripheral capillary network in the superficial layer. Some angiogenic 'buds' from the superficial layer grow downwards and vascularise the deep retinal layer [Gariano *et al*, 1994; Provis,

2001]. Glial astrocytes support and provide a framework for capillary development. Astrocyte invasion precedes retinal vascular development and also migrates radially [Ling and Stone, 1988; Provis *et al*, 1997; Chu *et al*, 2001]. Over time, the primary vascular plexus is remodelled and matures into a hierarchical vascular tree [Fruttiger, 2007].

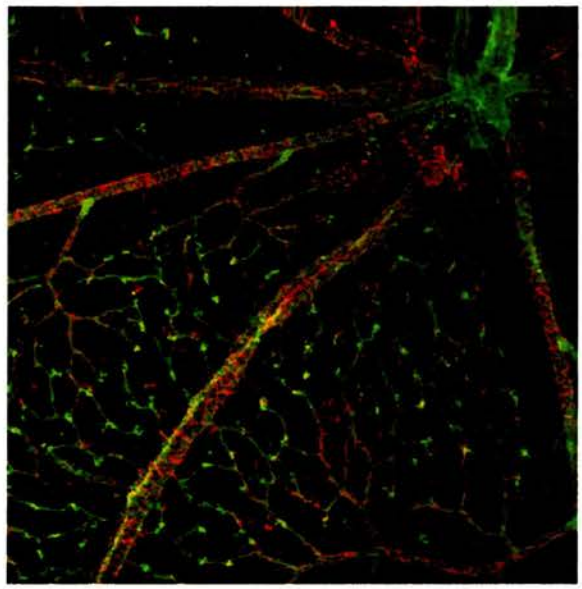


Figure 1.1 Intricate association between astrocytes (red) and blood vessels (green) in central area of rat pup retina. [picture taken by C.Dhaliwal and T.Gillespie]

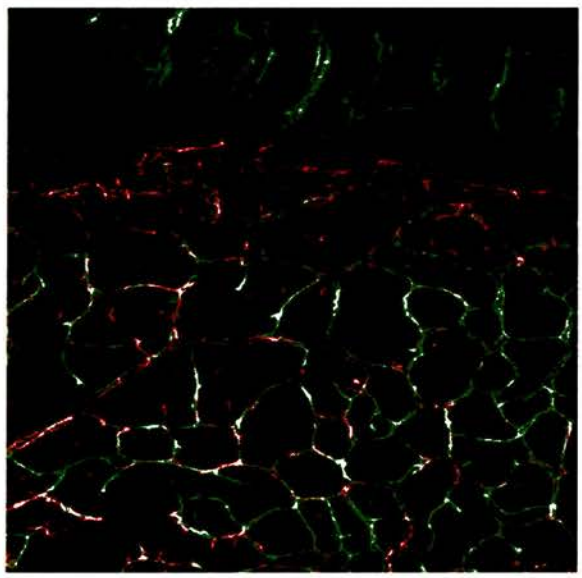


Figure 1.2 Astrocytes (red) precede developing blood vessels (green) to ora serrata (green superior edge). [picture taken by C.Dhaliwal and T.Gillespie]

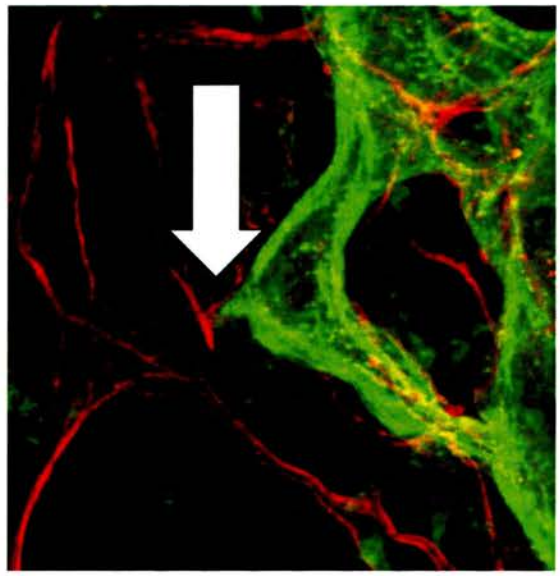


Figure 1.3 Angiogenesis-filopodia budding from existing vascular network into supportive astrocyte framework. [picture taken by C.Dhaliwal and T.Gillespie]

Vascular endothelial growth factor (VEGF) plays a key role in both normal angiogenesis and abnormal retinal blood vessel development. VEGF was initially identified as a 'permeability factor' in 1983 and in 1989 was identified as an endothelial cell mitogen and important in angiogenesis [Senger *et al*, 1983; Leung *et al*, 1989]. Six isoforms of VEGF exist (VEGF 121, 145, 165, 183, 189, 206), produced by alternative splicing of VEGF mRNA. Hypoxia inducible factor-1 (HIF-1) is a nuclear protein and transcription factor for VEGF. VEGF mRNA expression is increased in hypoxic conditions [Plate and Warnke, 1997].

Normal retinal angiogenesis on the superficial layer is driven by a zone of 'physiological hypoxia' in the region anterior to the developing vascular front [Chan-Ling *et al*, 1995]. Astrocytes and Muller cells secrete $VEGF_{165}$ in response to this local hypoxia resulting in endothelial cell proliferation and blood vessel development [Dorrell *et al*, 2002; Forsythe *et al*, 1996; Stone *et al*, 1995]. The new blood vessels in turn supply oxygen to the local tissue and this 'normoxia' negatively feedbacks to reduce $VEGF_{165}$ production [Stone *et al*, 1995]. The wave of physiological hypoxia then moves more peripherally and the cycle repeats until the ora serrata is reached.

1.2 Abnormal retinal blood vessel development

Infants born prematurely have an incompletely vascularised retina with a peripheral avascular zone. The more premature the baby is, the larger the avascular zone. ROP is recognised by specific morphological changes seen at the junction between the vascular and avascular retina.

ROP is described in terms of severity (stages 1-5), location (zones 1-3), extent of disease (1-12 clock hours) and presence or absence of 'plus' disease [The Committee for the Classification of Retinopathy of Prematurity, 1984, 2005]. In stage 1 ROP there is a demarcation line present between the central vascular and peripheral vascular retina. This develops into a ridge in stage 2 ROP. This ridge is associated with extra-retinal neovascularistion in stage 3 ROP. Stage 4 is partial retinal detachment and stage 5 is total retinal detachment and blindness. Stages 1 and 2 are considered to be 'mild' and mostly regress spontaneously. Stage 3 may regress or progress to stage 4 or 5. The zones are centred on the optic nerve. Zone 1 is the most posterior and is a circle of radius twice the optic nerve-to-macula distance. Zone 2 is an annulus with the inner border defined by zone 1 and the outer border defined as the distance from the optic nerve to the nasal ora serrata. Zone 3 is the residual temporal crescent of the retina (figure 1.4). Disease in zone 1 is high risk disease. The extent of ROP is defined in a circumferential pattern by clock hours of involvement with 12 o'clock being superior. 'Plus' disease refers to the appearance of the posterior blood vessels. It is said to be present when the posterior arterioles are tortuous and the posterior venules are dilated. It can progress to include iris vascular engorgement, vitreous haze and poor pupillary dilatation.

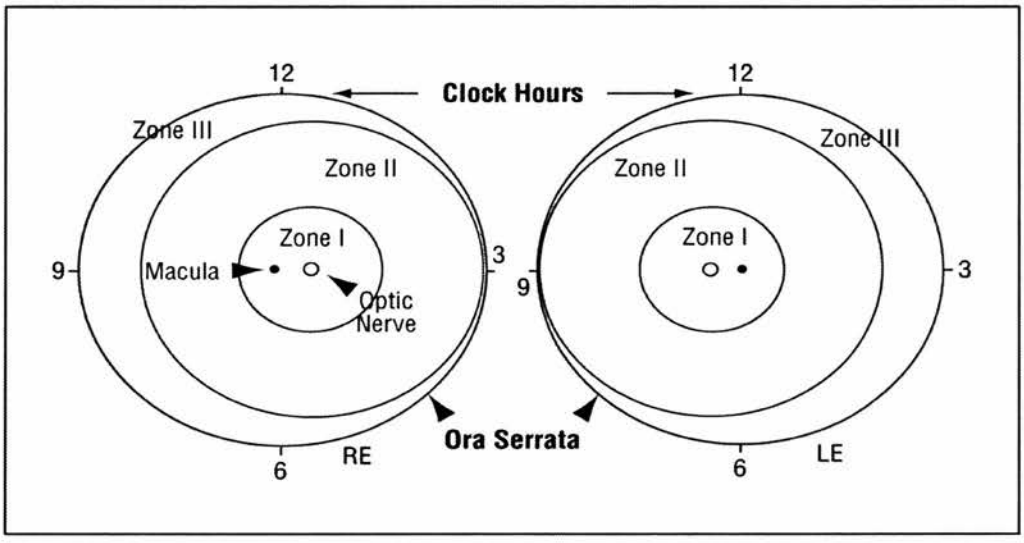


Figure 1.4 Scheme of retina of right eye (RE) and left eye (LE) showing zone borders and clock hours used to describe the location and extent of ROP (ICROP Revisited *Arch Ophthalmol*.2005:123:992)

The clinical histopathology of ROP is poorly understood. The demarcation line/ridge in stages 1-3 ROP originates from mesenchymal tissue and is composed of immature astrocytes [Fleck and McIntosh, 2008]. It is possible that these astrocytes lose their supportive function and allow extraretinal vascular growth into the vitreous which instead supports the immature vessels. The ridge also contains arteriovenous shunt vessels and posterior to the ridge are areas of capillary non-perfusion [Schulenburg and Tsanaktsidid, 2004]. Plus disease is thought to occur as a result of increased retinal blood flow to these arteriovenous shunts and could be caused by a direct effect of VEGF [Saunders *et al*, 1995; Schaffer *et al*, 1993].

One theory that is widely accepted to explain the development of ROP is that it is a two-phase disease [Smith, 2004]. Impaired growth of retinal vessels in phase 1 is followed by hypoxia-driven uncontrolled vessel growth in phase 2. VEGF is well accepted as playing a crucial role in both phases of the pathogenesis of ROP.

In phase 1, the 'physiological hypoxia' which drives normal fetal retinal vascularisation is suddenly arrested following preterm birth. The preterm infant is

exposed to breathing room air and often requires supplemental oxygen. *In utero* retinal development occurs at oxygen tensions of 2-3kPa and after birth the preterm infant will have circulating oxygen tensions of 6-10kPa [Fleck and McIntosh, 2008]. This relative 'hyperoxia' of the extrauterine environment suppresses VEGF expression and leads to cessation and subsequent regression of retinal vascular growth [Smith, 2004]. In the weeks following birth the preterm infant grows and matures and the peripheral avascular retina becomes increasingly metabolically active. Local tissue hypoxia in this region occurs as the vascular supply is insufficient for the needs of the developing retinal tissue. VEGF secretion is increased in response to local hypoxia and in turn triggers pathological neovascularisation which is the hallmark of phase 2 ROP and occurs when the infant is 32-24 weeks corrected gestational age (CGA) [Pierce *et al*, 1995].

This theory is however not universally accepted as some clinician scientists feel it is too simplistic and does not relate adequately to the clinical situation where there is a gradual progression from mild-moderate-severe ROP rather than a sudden 'phase 2' of neovascularisation and it does not explain why regression of ROP which is often seen clinically occurs.

1.2.1 VEGF

Animal studies have shown that VEGF plays a central role in ocular neovascularisation [Aiello *et al*, 1995; Robinson *et al*, 1996; Adamis *et al*, 1996, Donahue *et al*, 1996; Miller *et al*, 1994; Stone *et al*, 1996; Young *et al*, 1997]. Intravitreal injection of a VEGF inhibitor in mice (either VEGF antisense oligonucleotide or VEGF binding molecule) during phase 2 has been shown to significantly reduce retinal neovascularisation [Aiello *et al*, 1995; Robinson *et al*, 1996].

Clinical studies also support the importance of VEGF in retinal neovascularisation and elevated levels of VEGF have been measured in the vitreous from patients with diabetic proliferative retinopathy [Aiello *et al*, 1994; Adamis *et al*, 1994]. VEGF has also been isolated from the retina of a patient with ROP [Young *et al*, 1997].

The importance of VEGF and oxygen in ROP pathogenesis is accepted but other important molecules and pathways must also be involved as neovascularisation is still seen despite inhibition of VEGF and ROP is still seen clinically despite rigorous control of supplemental oxygen.

1.3 ROP screening

The purpose of screening is to identify those infants who will need therapy to prevent disease progression. Screening protocols differ geographically. In the UK, current guidelines recommend that all infants with birth weight (BW) <1501g and/or gestational age (GA) <31 weeks undergo screening for ROP [Royal College of Paediatrics and Child Health, 2008]. Fewer than 10% of infants screened will develop severe ROP warranting treatment [Dhaliwal *et al*, 2008].

Screening is carried out using binocular indirect ophthalmoscopy (BIO) with eyelid speculum and scleral indentation performed by an experienced ophthalmologist commencing when the infant is approximately 34 weeks CGA (or 4-5 weeks postnatal age) and continued weekly or fortnightly until complete retinal blood vessel development has occurred. It is recognised as being a painful and distressing procedure for infants [Dhillon *et al*, 1993].Wide-field digital retinal imaging (WFDRI) is another screening method. This is an attractive screening tool as trained staff can capture retinal images which can be interpreted by a remote ophthalmologist. There is still much controversy over which examination method is best.

ROP screening is a significant work-load for ophthalmologists and is a stressful examination for infants although remains vital to detect and treat disease. There is scope for further refinement of screening programmes.

1.4 ROP treatment

Destruction of the avascular retina with laser photocoagulation or cryotherapy causes involution of pathological vessels and regression of ROP. The multicentred trial of cryotherapy for ROP (cryo-ROP) found that treatment of the peripheral avascular

retina in infants with severe disease decreased the chance of retinal traction/detachment by 41% [Cryotherapy for ROP cooperative group, 1988]. Cryotherapy has been largely replaced by laser photocoagulation as the preferred treatment modality. Laser photocoagulation is better for treating posterior disease, better tolerated than cryotherapy and is associated with a better visual outcome [Ng *et al*, 2002; Connolly *et al*, 2002]. Treatment prevents blindness but ophthalmological status is not normalised as treatment is nonselective and destroys all retinal cell types [Cryotherapy for ROP cooperative group, 1988]. Children with previous ROP commonly have reduced visual acuity, visual fields and contrast sensitivity, refractive errors and strabismus [Larsson *et al*, 2003; Larsson *et al*, 2004; Larsson *et al*, 2005; Larsson and Holmstrom, 2006; O'Connor *et al*, 2002]. They are also at increased risk of retinal detachment in later life [Park *et al*, 2004].

Treatment was previously directed at infants with 'threshold' disease which refers to stage 3 ROP present in either zone 1 or zone 2, with at least 5 contiguous or 8 continuous clock hours of disease with the presence of plus disease [Cryotherapy for ROP cooperative group, 1988]. The Early Treatment for ROP (ETROP) study showed that earlier laser treatment for infants with type 1 disease further reduced the risk of severe visual morbidity [Early Treatment for ROP Cooperative Group, 2003]. Type 1 disease was defined as zone 1 ROP (any stage) with plus disease; zone 1, stage 3 disease with/without plus disease and zone 2 ROP (stage2/3) with plus disease [Early Treatment for ROP Cooperative Group, 2003]]. A reduction from 19.5% to 14.5% in an unfavourable grating visual acuity measurement and from 15.6% to 9.1% in an unfavourable structural outcome at 9 months was seen in infants treated with type 1 disease compared with the control group that was not treated until threshold ROP was reached [Early Treatment for ROP Cooperative Group, 2003]. Treatment is now recommended for all infants with type 1 disease [Royal College of Paediatrics and Child Health, 2008]. The presence of 'plus' disease is now the primary indication for laser therapy. Scleral buckling and or vitrectomy surgery may be performed for infants with stage 4/5 ROP but the outcome is generally poor [Shah *et al*, 2009].

An anti-VEGF aptamer and anti-VEGF antibody have been developed (bevacizumab) and are currently used for treating neovascularisation associated with age-related macular degeneration. They are administered by injection into the vitreous of the eye. Bevacizumab has been used with positive effect in some infants to treat ROP and clinical trials are planned to evaluate and formalise their use [Mintz-Hittner *et al*, 2008; Chung *et al*, 2007; Kusaka *et al*, 2008; Lalwani *et al*, 2008; Quiroz-Mercado *et al*, 2008].

Visual morbidity post ROP treatment would be reduced if a preventive therapy was available. Children with spontaneously regressed ROP also suffer visual morbidity and would therefore also benefit from a preventative therapy. Vitamin A is required by the retina for both low-light and colour vision. It is also essential for growth and development. Preterm infants have low levels of vitamin A and some studies have related this to an increased risk of developing ROP [Mactier and Weaver, 2005]. Vitamin A supplementation may therefore play a role in the prevention of ROP. Antioxidant factors have been investigated as possible preventative strategies. Metanalyses of clinical trials using antioxidant vitamin E therapy suggest this strategy is beneficial [Raju *et al*, 1997] but use of this therapy is not widespread mainly because one clinical trial showed an increase in sepsis and mortality following high-dose vitamin E therapy [Johnson *et al*, 1985].

1.5 Epidemiology of ROP

ROP, as it is known today, was first described by Terry in 1942 as a 'fibroblastic overgrowth of persistent vascular sheath behind each crystalline lens' [Terry, 1942]. Since this first description, the incidence of ROP has varied over time and across the world as a result of changes in clinical neonatal practice. Within the developed world there have been two notable epidemics [Campbell, 1951; Gibson *et al*, 1989; Gibson *et al*, 1990; Valentine *et al*, 1989].

The first epidemic was seen in the early 1950s and was associated with exposure to high oxygen concentrations for prolonged periods of time [Campbell, 1951]. Oxygen therapy became available in clinical practice just before and during the Second

World War. The first randomised controlled trial of oxygen therapy was published in 1956 and found that 'retrolental fibroplasia' was more commonly seen in infants exposed to liberal oxygen therapy compared to those exposed to oxygen therapy which was restricted to the lowest concentration necessary to avoid clinical cyanosis [Kinsey, 1956]. The epidemic ceased following controlled oxygen therapy and the incidence of childhood blindness due to 'retrolental fibroplasia' fell dramatically [Hatfield, 1972]. Unfortunately, this decline was accompanied by an increase in mortality of very low birth weight (VLBW) infants from respiratory distress syndrome [Avery, 1960] and an increase in morbidity from brain injury, [McDonald, 1963]. Subsequently, oxygen therapy remained controlled but was tailored to an infant's needs according to blood gas measurements.

The second epidemic developed in the late 1970s and early 1980s and was related to an increased survival of VLBW infants associated with advances in neonatal medicine [Gibson *et al*, 1989; Gibson *et al*, 1990; Valentine *et al*, 1989; Schalij-Delfos and Cats, 1997]. These advances included the introduction of antenatal corticosteroids, surfactant therapy, new methods of neonatal mechanical ventilation and continuous oxygen monitoring with intravascular and transcutaneous electrodes [Horbar *et al*, 1993; Vento *et al*, 2005; Richardson *et al*, 1998; Cooke, 2006]. Very premature infants were surviving but their inherent immaturity was making them at increased risk of developing ROP.

There is a marked variation in the incidence of ROP worldwide. Globally, approximately 50,000 children are blind as a result of ROP [Gilbert *et al*, 2005]. In industrialised countries with infant mortality rates (IMR) of <10 per 1000 live births, ROP accounts for between 6-20% of childhood blindness [Steinkuller *et al*, 1999; Gilbert *et al*, 1997; Fleck and Dangata, 1994; Goggin and O'Keefe, 1991]. In these countries, sight threatening ROP is predominantly seen in infants weighing <750g at birth and is uncommon in infants weighing >1000g [Lad *et al*, 2008]. The CRYO-ROP multicentre study found that 18% of infants weighing <1251g at birth developed stage 3 ROP and only 6% required treatment [Cryotherapy for ROP cooperative group, 1988]. In the United Kingdom, 5-8% of childhood vision

impairment in 1985-1990 resulted from ROP-induced partial or complete blindness and this incidence had decreased to 3% in 2000[Rahi and Cable, 2003; Rahi and Dezateux, 1998]. At least 17/233 (7%) preterm babies with stage 3 ROP identified in the UK over a 14 month period from 1997-1999 were blind in one or both eyes as a result of the disease [Haines *et al*, 2005].

ROP remains an important cause of childhood blindness in countries with IMRs of 10-60 per 1000 live births [Gilbert, 2008]. These 'middle-income' countries include the Former Socialist Economies, Latin America and the emerging powerful economic countries of China and India [Chaudhari *et al*, 2009]. This has been referred to as the 'third epidemic' of ROP [Gilbert *et al*, 1997]. In these countries there is a wide range of BWs and GAs of infants developing severe ROP suggesting that this epidemic combines features of both the first and second epidemics [Gilbert, 2008]. The 'third epidemic' is thought to result from higher rates of preterm birth, improved access to neonatal intensive care, increased neonatal exposure to ROP risk factors and lack of screening and treatment programs in these 'middle-income' countries.

1.6 Aetiology of ROP

ROP is a multifactorial disease. Risk factors for ROP have been identified predominantly from numerous case-control studies and cohort studies.

1.6.1 Main risk factors: low birth-weight, immaturity, oxygen exposure

Low birth weight, low gestational age and exposure to supplemental oxygen are the most widely and consistently reported risk factors for ROP. In most epidemiological studies, the association between birth weight and ROP is stronger than the association between GA and ROP [Cryotherapy for ROP cooperative group, 1988; Schaffer *et al*, 1993; Seiberth and Linderkamp, 2000].

As mentioned above, ROP was associated with excessive high concentration oxygen therapy shortly after the condition was first described. Since this link was established, there has been a lot of research focused on the role of oxygen in the pathogenesis of ROP. Neonatal units now administer controlled supplemental

oxygen to premature infants. Oxygen levels are monitored by intermittent blood gas sampling and by assessing arterial oxygen saturation by continuous pulse oximetry. Prolonged respiratory support and exposure of infants to relatively high pulse oximetry oxygen saturations have been associated with increased risk of ROP [Tin *et al*, 2001; Anderson *et al*, 2004; Chow *et al*, 2003]. In addition, infants exposed to greater variations in oxygen levels during the first 2 weeks of life have also been shown to be at risk of developing ROP [Cunningham *et al*, 1995]. Recent case series where infants were exposed to stable, tightly controlled, relatively low oxygen saturations on pulse oximetry have consistently reported low incidences of severe ROP [Chow *et al*, 2003]. Importantly though, a large randomized trial comparing target oxygen saturations of 85-89% or 91-95% in preterm infants from 24 to 28 weeks gestation yielded a worrying result. There was an increase in mortality (death before discharge) in the lower-oxygen-saturation group [Carlo *et al*, 2010]. A large multi-centre randomised controlled trial is currently underway in the United Kingdom to try and determine the optimal oxygen saturation range for preterm infants (www.npeu.ox.ac.uk/boost). There is an urgent need to clarify the appropriate oxygen saturation range in order to minimize ROP but without increasing adverse outcomes.

1.6.2 Body growth and growth factors

Premature infants are born with incompletely vascularised retinas which must complete development postnatally. Low BW and low GA have been most consistently and directly reported as key risk factors for ROP. Taken together, it is therefore conceivable that nutritional status and availability of growth factors may influence postnatal retinal vascularisation.

Premature infants who are born small-for-gestational age (SGA, BW<10^{th} percentile) are at increased risk of neonatal morbidity and mortality [Bardin *et al*, 1997; Beeby, 1998; Tyson *et al*, 1995; Kok *et al*, 1998; Bernstein *et al*, 2000; Regev and Reichman, 2004; Ley *et al*, 1997]. They comprise a heterogeneous group of infants. The majority of infants in the western world born SGA are as a result of placental insufficiency causing intrauterine growth restriction (IUGR). Other SGA infants may have a fetal or maternal reason for being small such as a congenital or genetic defect,

infectious disease, multiple birth or they may be constitutionally small due to short parental height. Several studies have reported that SGA infants are at increased risk of developing ROP [Darlow *et al*, 2005; Allegaert *et al*, 2003; Regev *et al*, 2003; Bardin *et al*, 1997].

Postnatal growth retardation is inevitable in preterm infants [Cooke *et al*, 2004; Embleton *et al*, 2001]. Recent research has highlighted that poor postnatal weight gain of VLBW infants is an independent risk factor for ROP [Wallace *et al*, 2000; Lofqvist *et al*, 2006; Hellstrom *et al*, 2009; Hall *et al*, 1995]. In addition, a low weight at 32 weeks corrected gestational age and low weight at school age has been shown to be related to poor childhood visual acuity and poor visual perception [Hok-Wikstrand *et al*, 2010]. This highlights that rate of neonatal body growth has an important impact on childhood visual development.

1.6.2.1 Insulin-like growth factor 1 (IGF-1)

With the emergence of the importance of body growth and rate of growth on the development of ROP, research has recently been directed at finding a mechanism to explain this clinical association. IGF-1 has been earmarked as a key molecule. IGF-1 is a peptide and is essential for body growth. It was originally discovered as a mediator of growth hormone actions but it is also an important regulator of cell metabolism, differentiation and survival. IGF-1 is secreted by most fetal tissues and after birth is produced predominantly by the liver [Holly and Perks, 2006]. Nutrition together with pituitary growth hormone regulates the IGF system. Serum IGF-1 concentration rises with increasing gestational age and increasing fetal size. Levels increase significantly during the third trimester [Arosio *et al*, 1995; Pirazzoli *et al*, 1997; Holmes *et al*, 1997; Langford *et al*, 1998; Reece *et al*, 1994]. The effects of IGF-1 are mediated by the IGF-1 receptor (IGF-1R). This is a classical transmembrane tyrosine kinase cell surface receptor. There are six different IGF binding proteins (IGFBPs) and the principal carrier and regulator of the bioavailability of IGF-1 is IGF binding protein 3 (IGFBP-3).

Animal studies have shown that IGF-1 is important for normal retinal vascular growth as IGF-1 knockout mice show slower retinal vascular development than wild-

type controls. IGF-1 has also been shown to control maximum VEGF activation of the Akt endothelial cell survival pathway [Hellstrom *et al*, 2001]. In the proliferative phase (phase 2), administration of an IGF-1 receptor antagonist in mice was found to reduce retinal neovascularisation without influencing VEGF levels [Smith *et al*, 1999]. Transgenic mice with vascular endothelial cell specific knockout of the IGF-1 receptor showed suppression of retinal neovascularisation compared to controls following exposure to a high oxygen profile known to induce proliferative retinal changes [Kondo *et al*, 2003].

Clinically, serum IGF-1 levels in preterm infants of the same gestational age were significantly lower in infants who went on to develop ROP [Hellstrom *et al*, 2003]. Weekly postnatal weight gain measurements together with serum IGF-1 levels have been used to predict infants at risk of severe ROP [Lofqvist *et al*, 2006; Hellstrom *et al*, 2009]. Young adults who were born SGA as a result of IUGR (with abnormal fetal blood flow) have reduced retinal vascularisation with fewer branching points on blood vessels [Hellstrom *et al*, 2004].

Thus both animal and human studies have highlighted that IGF-1 plays a role in normal retinal blood vessel development and in abnormal neovascularisation seen in severe ROP. It has been suggested that one of the ways that IGF-1 may regulate retinal neovascularisation is through control of VEGF activation of p44/42 mitogen-activated protein kinases (MAPK) [Hellstrom *et al*, 2001; Smith *et al*, 1999]. It has been proposed that sufficient serum levels of IGF-1 are required for appropriate VEGF-induced vessel growth in normal retinal vascular development. The following theory has been developed to explain the roles of IGF-1 and VEGF in ROP. After preterm birth, the supply of nutrients and growth factors from the placenta is suddenly lost and serum IGF-1 levels decrease and remain low until the neonatal liver takes over production [Langford *et al*, 1998; Hikino *et al*, 2001]. Low IGF-1 together with low VEGF (due to relative 'hyperoxia' of extrauterine environment) cause suppression of normal vessel growth in phase 1. Over time, as the infant grows and matures, IGF-1 levels slowly rise and local retinal hypoxia leads to increased expression of VEGF. It is thought that phase 2 occurs when IGF-1 levels reach a

certain 'threshold' concentration that is required to permit VEGF to work maximally and stimulate the abnormal blood vessel growth which is the hallmark of phase 2 ROP [Chen and Smith, 2007].

1.6.3 Other risk factors

Male infants are at increased risk of developing severe ROP [Darlow *et al*, 2005; Nodgaard *et al*, 1996; Todd *et al*, 1990]. Infants who have had blood transfusions or erythropoietin therapy are at increased risk of ROP [Cooke *et al*, 1993; Hesse *et al*, 1997]. Genetic factors may play a role in a subset of infants with mutations in the Norrie disease gene [Shastry *et al*, 1997; Hiraoka *et al*, 2001]. Other reported contributing factors include hyperglycaemia [Ertl *et al*, 2006], sepsis [Liu *et al*, 2005; Bharwani and Dhanireddy, 2008], parenteral nutrition [Shohat *et al*, 1983], hypo/hypercarbia [Ben *et al*, 1988], patent ductus arteriosus, necrotising enterocolitis and intraventricular haemorrhage [Arroe and Peitersen, 1994]. With many of these factors, it is unclear if they are true independent risk factors for ROP or simply indicators of the compromised health of the neonate.

Studies on light reduction have concluded that reduction of light exposure does not reduce the progression of ROP [Reynolds *et al*, 1998]. Maternal smoking may have an inhibitory effect against developing severe ROP, perhaps relating to nitric oxide exposure, but further research is required [Hirabayashi *et al*, 2010].

1.7 Experimental models of ROP

Models of ROP have yielded much of what is currently known on the molecular and cellular mechanisms underlying the disease. Rodents such as mice and rats are predominantly used to study the pathogenesis of ROP. Murine systems are popular due to the possible genetic manipulations available in mice. Rodent pups are born with no retinal blood vessels (and are therefore at an equivalent stage of development as a 16 week human fetus) and vascularisation occurs during the first 2 weeks of life [Henkind, 1967; Cairns, 1959]. The pattern of vascularisation has some similarities to that of the human. Exposure to oxygen following birth is used to induce abnormal retinal vascularisation akin to human ROP in rodent pups [Patz, 1954]. There are many different oxygen profiles used [Ricci and Caolgero, 1988; Ventresca *et al*,

1990; Penn *et al*, 1994]. Popular protocols used in the mouse include exposure to high oxygen levels (75%) from postnatal day 7 until day 12 then return to room air until day 17-21 [Smith *et al*, 1994]. Penn *et al* developed many of the rat models using alternating daily exposure to 40/80% and 50/10% oxygen for the first 14 days of life [Penn *et al*, 1994; Penn and Johnson, 1993]. The 50/10% oxygen exposure regimen has become a widely accepted model of ROP. Retinal neovascularisation has also been induced in rodents secondary to early acidosis [Holmes and Duffner, 1999].

These models although effective at inducing abnormal retinal vascularisation are very unphysiological and are not a true representation of a preterm infant's oxygen exposure. This led the Edinburgh group to develop 'the Edinburgh rat model of ROP' [McColm *et al*, 2000]. The fluctuating oxygen profile over the first 14 days of life from a preterm infant who developed threshold ROP was taken. The oxygen profile was recorded on a minute-minute basis via a transcutaneous oxygen probe and staff aimed to keep the infant's oxygen levels between 6-10kPa (mean 8kPa). This profile was converted into an equivalent inspired oxygen profile for a rat. Pups were exposed to this computer-controlled profile over the first 14 days of life. At 14 days, the pup retinas showed significantly larger avascular areas than room air controls. No neovascularisation was observed. The strength of this model is that it is very physiological and a more true representation of the oxygen exposure of a preterm infant.

Several animal models have been developed to study the relationship between body growth and retinal vascularisation. Rat pups can be 'cross-fostered' after birth so one dam can raise pups from two originally separate litters. These pups grow more slowly in the postnatal period than those raised in 'normal-sized' litters. On exposure to high levels of oxygen the growth retarded pups from the larger litters have shown more severe abnormal retinal neovascularisation [Holmes and Duffner, 1996; Zhang *et al*, 2001]. These animal models have been created by manipulating postnatal growth as weight at birth of the rat pups was not different.

1.8 Summary

The World Health Organisation's Vision 2020 programme identifies ROP as an 'avoidable disease' [Gilbert and Foster, 2001]. Significant visual impairment as a result of ROP affects the motor, language, conceptual and social development of a child and their quality of life [Mets, 1999; Quinn *et al*, 2004; Halsey *et al*, 1993; Tamai and Majima, 1996; Msall *et al*, 2004. It also carries a high financial cost for society. Early detection and treatment to prevent blindness is vital. This is a worldwide challenge as the disease is now becoming more prevalent in developing countries.

The role of body growth and growth factors in the pathogenesis of ROP remains poorly understood but is an exciting area as there is potential to develop preventative strategies based on nutrition. This is the overall theme of my work. I start my thesis with an epidemiological study reporting on the incidence of ROP in Lothian (Chapter 2). As I am interested in the effect of growth on ROP development, I compared the incidence of ROP in SGA and appropriate-for-gestational age (AGA) infants within the Lothian population and I report these findings in Chapter 3. I manipulated the 'Edinburgh rat model of ROP' in order to study the effect of nutrition on retinal vascular development within this animal model. Chapter 4 reports on my findings related to rat growth, serum IGF-1 and retinal vascularisation. Alongside this work, I carried out a randomised clinical trial to validate the use of WFDRI as a screening modality for ROP detection. Chapters 5 and 6 report the trial findings. This work links into my theme of 'nutrition' as WFDRI techniques are being developed to measure human retinal vascular development in SGA and poor postnatal weight-gain infants. Chapter 7 summarises all my findings and outlines scope for future work.

CHAPTER 2

THE INCIDENCE OF RETINOPATHY OF PREMATURITY IN LOTHIAN, SCOTLAND

Dhaliwal C, Fleck B, Wright E, Graham C, McIntosh N.
Incidence of retinopathy of prematurity in Lothian, Scotland, from 1990 to 2004.
Arch Dis Child Fetal Neonatal Ed. 2008 Nov;**93**(6):F422-6.

2.1 Introduction

Since retinopathy of prematurity (ROP) was first described in 1942 [Terry, 1942], there have been two notable epidemics within the developed world [Campbell, 1951; Gibson *et al*, 1989; Gibson *et al*, 1990; Valentine *et al*, 1989]. The first was seen in the early 1950s and was associated with unmonitored supplemental oxygen for preterm infants [Campbell, 1951]. The second epidemic developed in the 1980s and was related to increased survival of very low birth weight (VLBW) infants associated with advances in neonatal medicine [Gibson *et al*, 1989; Gibson *et al*, 1990; Valentine *et al*, 1989; Schalij-Delfos and Cats, 1997]. The subsequent trends in incidence of ROP have been the subject of much debate. There is widespread variability in the reported incidence of ROP in hospital cohorts and population-based studies. Some centres in the developed world reported a decline in the incidence of ROP in the 1990s and early 2000s [Hussain *et al*, 1999; Bullard *et al*, 1999; Rowlands *et al*, 2001; Keith and Doyle, 1995] but other centres in the developed world also reported an increase in the incidence of ROP [Valentine *et al*, 1989; Gibson *et al*, 1990; Schalij-Delfos and Cats, 1997]. This variability in incidence and severity of ROP reflects differences in case demographics, selection variability, observer bias and outcome measures together with differences in neonatal care provided. Large-scale population-based studies on ROP have been limited and these report the longer term trends in incidence most accurately [Darlow *et al*, 2003; Ng *et al*, 1988; Darlow, 1988; Todd *et al*, 2007; Chiang *et al*, 2004; Hameed *et al*, 2004].

2.2 Aim

To report the trends in incidence of ROP within the Lothian region of South East Scotland over a 15-year period from 1990-2004.

2.3 Methods

2.3.1 Subjects

This was a prospective observational cohort study. The study population consisted of all infants eligible for eye screening who were born to mothers resident within the Lothian region of South East Scotland during the study period of January 1st 1990-December 31st 2004. All infants born with gestational age (GA)<32 weeks and/or

birth weight (BW)<1500 grams who survived until eye screening commenced were eligible. Eligible Lothian babies born outside Lothian and transferred back into Lothian during eye screening were included.

During the study period infants were born at one of three hospitals:

The Simpson Memorial Maternity Pavilion / The New Royal Infirmary, Edinburgh

This is the level 3 South East Scotland Regional Neonatal Unit with over 6000 live births per year. Approximately 520 infants are admitted to the neonatal unit each year. The hospital moved to the site of the New Royal Infirmary, Edinburgh in summer 2002.

The Eastern General Hospital, Edinburgh

This hospital closed in 1998 but before then had a level 2 neonatal unit with approximately 2200 live births and 250 babies admitted to the neonatal unit each year. After closure, babies were delivered at the Simpson's or New Royal Infirmary, Edinburgh.

St. Johns Hospital, Livingston

This hospital has a level 2 neonatal unit with approximately 2400 live births 170 admissions to the neonatal unit each year.

Nursing staff in these hospitals referred all eligible babies to the ophthalmology team. Ophthalmologists recorded the date of birth, sex, gestational age, birth weight and maximum severity of ROP reached in any one eye for every baby examined. This information was prospectively stored on a database. Neonatal protocols were similar in the 3 units.

2.3.2 Lothian population epidemiological data

Epidemiological data were obtained from the Information Services Division (ISD) of the Scottish Health Service who accessed the Scottish Morbidity Record (SMR02). The ISD figures differ from our Lothian hospital data and our results are based on both sources. Unfortunately we were unable to cross-check our data with ISD due to

patient confidentiality and data protection legislation, thus a small discrepancy remains. For the purposes of our study, survival was defined as survival to 42 weeks corrected gestational age.

2.3.3 Eye examination schedule

Screening and treatment (if required) was carried out by 2 dedicated paediatric ophthalmologists (Dr Brian W. Fleck and Dr Elizabeth Wright). Eligible infants were first examined at 4-6 weeks chronological age, or 34 weeks corrected age, whichever was earlier. Screening was continued fortnightly until full retinal vascularisation. Examinations were performed weekly if 'prethreshold' disease was found (see below for definition).

2.3.4 Eye examination technique

Pupils were dilated with topical phenylephrine 2.5% and tropicamide 0.5% applied 60 minutes and 30 minutes prior to eye examination. Indirect ophthalmoscopy was performed using a binocular indirect ophthalmoscope and 28-dioptre lens. A lid speculum and scleral indenter were routinely used. The entire retina was examined, including the periphery throughout 360 degrees. Retinopathy was graded according to The International Classification of ROP [The Committee for the Classification of Retinopathy of Prematurity, 1984]. The stage of ROP (1-5), zone of vascularisation, number of clock hours of ROP and presence or absence of 'plus' disease was documented in both the eye logbook and patient medical notes. 'Plus' disease represented significant dilatation and tortuosity of posterior pole blood vessels [Cryotherapy for ROP cooperative group, 1988]. 'Threshold' ROP referred to 5 or more contiguous or 8 or more cumulative clock hours of stage 3 ROP in zones 1 or 2 in the presence of 'plus' disease [Cryotherapy for ROP cooperative group, 1988]. 'Prethreshold' disease consisted of any stage 3 disease less extensive than threshold, or stage 2 disease in the presence of 'plus' [Cryotherapy for ROP cooperative group, 1988]. All eyes examined with 'threshold' ROP were treated with cryotherapy in 1990-1991 and with diode laser therapy from 1992 onwards. From January 2005 new treatment criteria were used following the publication of the ETROP study [Early Treatment For Retinopathy Of Prematurity Cooperative Group, 2003]. The study

period therefore terminated at the end of 2004. Each hospital throughout the period of study used oxygen saturation monitor thresholds of 86-94%.

2.3.5 Statistical Analysis

The maximum severity of ROP in either eye for an individual infant was recorded. The 15-year study period was divided into three 5-year epochs: 1990-1994, 1995-1999, and 2000-2004. Statistical analysis was performed using the GraphPad InStat programme (GraphPad Software, California, USA). Contingency tables were analyzed using the chi-square test (X^2) and chi-square test for trend (X^2 trend). As birth weights and gestational ages did not to follow a normal distribution, Mann-Whitney tests were used to compare the missing eligible babies with those of the study population. The Kruskal-Wallis test was used to compare the birth weights and gestational ages of the study population in each of the three epochs. In all cases a p-value <0.05 was taken to indicate statistical significance.

The Lothian Research Ethics Committee was contacted and declared that no ethical approval was required for this research.

2.4 Results

2.4.1 Lothian population epidemiological data (Table 2.1)

The total population of Lothian increased steadily from approximately 745,000 in 1990 to 790,000 in 2004. There were approximately 9800 live births in 1990 which decreased over the years to 8300 in 2004.

The proportion of babies born with BW<1500g and/or GA<32 weeks that survive to 42 weeks corrected gestational age (CGA) has increased from 1990-2004 ($p<0.001$ using chi-square test for trend). This increase in survival is evident despite the proportion of these babies being born remaining unchanged ($p=0.271$ using chi-square test).

Table 2.1: Lothian population epidemiological data (supplied by ISD Scotland)

Time periods	LB	LB BW<1500g and/or GA<32wks (% of LB)	LB BW<1500g and/or GA<32wks admitted to Lothian neonatal units	LB BW<1500g and/or GA<32wks surviving to CGA 42wks (% of LB BW<1500g and/or GA<32wks)*
1990-1994	47,937	701 (1.5%)	637	586 (84%)
1995-1999	44,410	596 (1.3%)	547	518 (87%)
2000-2004	40,833	587 (1.4%)	564	532 (91%)
Total	133,180	1,884 (1.4%)	1,748	1,636 (87%)

LB=live births

* X^2 trend shows evidence towards increased survival 1990-2004 ($p<0.001$)

2.4.2 Study population-from Lothian hospitals data

During the study period, there were 1450 eligible babies registered for eye screening. 77 (5%) were discharged home prior to eye screening, or failed to attend outpatient eye screening, and there were insufficient medical records from 10 (0.7%) babies. Thus complete data was available from 1363 infants (1363/1450, 94% of eligible population). ISD report a total of 1636 babies from 1990-2004 with BW<1500g and/or GA<32wks where the baby survived to CGA 42wks or more (table 1). Therefore, a discrepancy of 186 babies remains between our hospital data and ISD data. We were unable to access details on these babies due to patient confidentiality and data protection legislation and therefore could not crosscheck our hospital records with ISD. We feel that our numbers are likely to be more accurate than ISD figures as ISD obtain their data from medical coding whereas our figures are based on raw data obtained from babies' eye examinations.

The median gestational age for the Lothian hospitals study population (1363) was 29 weeks, (interquartile range 28-31) and median birth weight was 1240g, (interquartile range 965-1490). The study population comprised 54% males. The median gestational age of the missing eligible babies (enough data only from 77 discharged babies) was 31 weeks, (interquartile range 30-32) and median birth weight was 1467g, (interquartile range 1340-1675). Using a Mann-Whitney test we found that the 77 babies had a significantly higher gestational age [$p<0.001$] and also a higher birth weight [$p<0.001$]. These baseline characteristics of the missing eligible babies

were expected as only the more mature and heavier babies would have been able to be discharged prior to commencement of eye screening.

The baseline characteristics of infants in the three time epochs: 1990-1994, 1995-1999 and 2000-2004 were also calculated. There was no evidence of statistically significant differences in birth weight (Kruskal-Wallis p=0.48) or gestational age at birth (Kruskal-Wallis p=0.09) between the three cohorts.

2.4.3 Incidence and severity of ROP in Lothian hospitals study population (Table 2.2)

One baby in 1999 developed stage 4 ROP after laser treatment. No babies developed stage 5 ROP during the study period. The heaviest baby treated weighed 1190g at birth and the most mature baby treated was 30 weeks gestation at birth.

Table 2.2: Incidence of ROP in Lothian hospitals study population (1363 babies)

	Total number of babies screened for ROP	Number of babies with any degree of ROP (% of total number)*	Number of babies with severe ROP (% of total number)$	Number of babies treated for ROP (% of total number)#
1990-1994	442	96 (22%)	53 (12%)	41 (9%)
1995-1999	447	94 (21%)	39 (9%)	21 (5%)
2000-2004	474	81 (17%)	45 (9%)	24 (5%)

Severe ROP=stage 3 or greater ROP

*no evidence of a statistically significant difference (X^2=3.628, p=0.16; X^2trend=3.13, p=0.08)

$ no evidence of a statistically significant difference (X^2=2.872, p=0.24; X^2trend=1.52, p=0.22)

evidence of a statistically significant difference (X^2=9.789, p<0.01; X^2trend=6.686, p<0.01)

2.4.4 Incidence and severity of ROP in Lothian hospitals study population for birth weight categories (Tables 2.3, 2.4)

From Lothian hospitals study population data, the total number of babies with BW<1500g who underwent eye screening was 1032 from 1990-2004. The remaining 331 babies had gestational ages <32 weeks but birth weights >1500g and were not included in this analysis. A trend towards a greater proportion of babies with

BW<750g having no ROP (p=0.03) was observed and a reduced proportion of these babies required treatment (p<0.01). The incidence of severe ROP in babies with BW 1000-1249g and 1250-1499g was consistently low and there was a trend towards fewer 1250-1499g birth weight babies having any ROP (p=0.04). No statistically significant trends from 1990-2004 were observed for babies with BW 750-999g and 1000-1249g.

Table 2.3: Incidence of ROP in Lothian hospitals study population in babies with BW<750g and BW 750-999g.

	Total babies screened in Lothian		Babies with no ROP (% of babies screened)		Babies with stages 1,2 ROP (% of babies screened)		Babies with stage 3 ROP-untreated (% of babies screened)		Babies with stage 3 ROP-treated (% of babies screened)	
	BW <750g	BW 750-999g	BW <750g^	BW 750-999g	BW <750g	BW 750-999g	BW <750g	BW 750-999g	BW <750g#	BW 750-999g
1990-1994	35	92	6 (17%)	50 (54%)	6 (17%)	16 (17%)	4 (11%)	7 (8%)	19 (54%)	19 (21%)
1995-1999	50	61	9 (18%)	37 (61%)	15 (30%)	15 (25%)	11 (22%)	4 (7%)	15 (30%)	5 (8%)
2000-2004	48	83	18 (38%)	51 (61%)	7 (15%)	16 (19%)	11 (23%)	6 (7%)	12 (25%)	10 (12%)

^linear trend towards increased proportion of babies with no ROP

(X^2=6.489, p=0.04; X^2trend=5.050, p=0.03)

#linear trend towards reduced proportion of babies treated

(X^2=8.418, p=0.01; X^2trend=7.148, p<0.01)

Table 2.4: Incidence of ROP in Lothian hospitals study population in babies with BW 1000-1249g and BW 1250-1499g.

	Total babies screened in Lothian		Babies with no ROP (% of babies screened)		Babies with stages 1,2 ROP (% of babies screened)		Babies with stage 3 ROP-untreated (% of babies screened)		Babies with stage 3 ROP-treated (% of babies screened)	
	BW 1000-1249g	BW 1250-1499g	BW 1000-1249g	BW 1250-1499g^	BW 1000-1249g	BW 1250-1499g	BW 1000-1249g	BW 1250-1499g	BW 1000-1249g	BW 1250-1499g
1990-1994	97	101	81 (84%)	95 (94%)	13 (13%)	5 (5%)	0 (0%)	1 (1%)	3 (3%)	0 (0%)
1995-1999	104	126	83 (80%)	121 (96%)	17 (16%)	5 (4%)	3 (3%)	0 (0%)	1 (1%)	0 (0%)
2000-2004	118	117	106 (90%)	116 (99%)	7 (6%)	0 (0%)	3 (3%)	1 (1%)	2 (2%)	0 (0%)

^ linear trend towards increased proportion of babies with no ROP

(X^2=4.301, p=0.12; X^2trend=4.224, p=0.04)

2.4.5 Incidence and severity of ROP in Lothian hospitals study population for gestational age categories (Tables 2.5, 2.6)

From Lothian hospitals population data, the total number of babies with GA<32wks who underwent eye screening was 1218 from 1990-2004. Similar trends were observed when considering the babies in GA categories (tables 2.5, 2.6)

Table 2.5: Incidence of ROP in Lothian hospitals study population in babies with GA</=24wks and GA 25-26wks.

	Total babies screened in Lothian		Babies with no ROP (% of babies screened)		Babies with stages 1,2 ROP (% of babies screened)		Babies with stage 3 ROP (untreated) (% of babies screened)		Babies with stage 3 ROP (treated) (% of babies screened)	
	GA </=24wks	GA 25-26wks	GA </=24wks^	GA 25-26wks*	GA </=24wks	GA 25-26wks	GA </=24wks	GA 25-26wks	GA </=24wks	GA 25-26wks#
1990-1994	13	57	0	23 (40)	2 (15)	8 (14)	1 (8)	6 (11)	10 (78)	20 (35)
1995-1999	18	43	0	7 (16)	5 (28)	18 (42)	6 (33)	7 (16)	7 (39)	11 (26)
2000-2004	21	64	5 (24)	28 (44)	4 (19)	15 (23)	2 (10)	10 (16)	10 (48)	11 (17)

^linear trend towards increased proportion of babies with no ROP out of babies screened 1990-2004 (X^2-8.166, p=0.02; X^2trend=6.285, p=0.01)

*evidence of a statistically significant change in proportion of babies with no ROP out of babies screened 1990-2004 but no evidence of a linear trend (X^2=9.441, p<0.01; X^2trend=0.231, p=0.63)

#linear trend towards reduced proportion of babies treated out of babies screened 1990-2004 (X^2=5.071, p=0.08; X^2trend=5.066, p=0.02)

Table 2.6: Incidence of ROP in Lothian hospitals study population in babies with GA 27-28wks and GA 29-31wks.

	Total babies screened in Lothian		Babies with no ROP (% of babies screened)		Babies with stages 1,2 ROP (% of babies screened)		Babies with stage 3 ROP (untreated) (% of babies screened)		Babies with stage 3 ROP (treated) (% of babies screened)	
	GA 27-28wks	GA 29-31wks	GA 27-28wks^	GA 29-31wks	GA 27-28wks	GA 29-31wks	GA 27-28wks	GA 29-31wks	GA 27-28wks#	GA 29-31wks
1990-1994	97	230	64 (66)	215 (94)	19 (20)	13 (6)	3 (3)	2 (1)	11 (11)	0
1995-1999	75	264	55 (73)	245 (93)	14 (19)	18 (7)	4 (5)	0	2 (3)	1 (<1)
2000-2004	90	246	74 (82)	233 (95)	7 (8)	10 (4)	6 (7)	3 (1)	3 (3)	0

^linear trend towards increased proportion of babies with no ROP out of babies screened 1990-2004 (X^2=6.355, p=0.04; X^2trend=6.338, p=0.01)

#linear trend towards decreased proportion of babies treated out of babies screened 1990-2004 (X^2=7.389, p=0.04 using a Fishers exact test due to small expected counts; X^2trend=5.353, p=0.02

2.5 Discussion

The trends in the incidence of ROP within the developed world since the early 1990s have been the cause of much debate. Some centres have reported an increase in the incidence of ROP (thought to be a reflection of improved survival rates for premature infants), whilst other centres have reported a decrease in incidence (thought to be due to improved neonatal care) [Valentine *et al*, 1989; O'Connor *et al*, 2003; Hussain *et al*, 1999; Chow *et al*, 2003; Wallace *et al*, 2007; Wright *et al*, 2006; Arroe and Peitersen, 1994; Keith *et al*, 1995]. Population studies over a long period of time provide the most robust evidence of epidemiological changes. Our prospective population based study has found a significant reduction in the number of babies treated for ROP from 1990-2004. A reduction in the overall incidence of any degree of ROP and severe ROP was also observed. We have also seen an increase in overall survival in babies with BW<1500g or GA<32wks.

Our observed changes are based on a complete population rather than single hospital data and therefore referral and inclusion bias should be eliminated. One of the key

strengths of this study is that inter observer variation should be minimal for detection of severe ROP as only 2 examiners examined the population over the 15-year period and both were experienced paediatric ophthalmologists. Observer bias has been shown to be one of the key causes of inter-centre variation in the incidence of ROP [Darlow *et al*, 2008]. The methods of ophthalmoscopic examination for ROP and the classification system used were the same for the whole study period. Oxygen saturation monitors were introduced in the late 1990s and the target limits have always been 86-94%. Arterial catheters were infrequently used throughout the study period with the target partial pressure of oxygen being 6-10kpa. We have not separately analysed mild stages (stage 1 or 2) of ROP, as this data is less robust due to likely inherent observer error in scoring these lesser grades. There may have been an under-reporting of 'any degree of ROP' and a related over-reporting of 'no ROP' as a consequence of failure to detect minor degrees of ROP. The context of this prospective data acquisition was as a clinical service rather than a precisely defined epidemiological project as in other studies [Ng *et al*, 1988]. We did not formally record ethnicity but results from the 2001 census in Scotland report the Scottish population as being 98% Caucasian, 1.4% Asian and 0.6% Afro-Caribbean and other ethnic background [The General Register Office for Scotland, 2007]. There was minimal movement of babies between different centres.

Recent population studies report differing trends in the incidence of ROP. I shall discuss studies that report on the incidence of severe ROP in the developed world as this data is likely to be more clinically relevant to our study population. I shall also only discuss studies pre-2005 as many centres in the developed world changed their clinical practice following the publication of the ET-ROP trial and this will likely affect the incidence of treated ROP reported [Early Treatment For Retinopathy Of Prematurity Cooperative Group, 2003].

Lee *et al* reported an 11% incidence of severe ROP and 3% incidence of treated ROP in Canada during 1996-1997 [Lee *et al*, 2000]. These incidences are very similar to our reported ones during 1995-1999 and probably reflect similar neonatal practices. Larsson *et al* compared ROP rates during 1988-1990 (260 infants) and 1998-2000

(253 infants) in Sweden and found no change in the incidence of severe ROP in infants with BW<1500g [Larsson *et al*, 2002]. Similarly, Lundqvist *et al* studied a population in southern Sweden from 1995-2004 and despite an increase in survival of premature infants <32 wks there were no changes in the incidence of severe ROP [Lundqvist *et al*, 2009]. Todd *et al* studied the incidence and treatment of severe ROP in New South Wales and the Australian Capital Territory from 1992-2002 [Todd *et al*, 2007]. They found a significant increase in severe ROP in infants </=24wks gestation (42% to 54%) together with an increase in treatment for severe ROP (19% to 24%). In infants of 25-26wks gestation there was a significant decrease in severe ROP (26% to 19%) and there was no change in 27-29 wks gestation infants. Hameed *et al* reported on severe ROP in 505 infants in Leicestershire, UK from 1990-1999 in infants with BW</=1250g [Hameed *et al*, 2004]. They found an increase in severe ROP from 4% in 1990-1994 to 12% in 1995-1999. This increase in severe ROP was also observed in infants with BW<750g. This is very different to our findings as we observed a reduction in the incidence of severe ROP in babies <750g. The reason for the different outcomes is unknown but the incidence of severe ROP may be influenced by variations in population ethnicity and relatively minor changes in neonatal care policies and practice.

The papers discussed above are all population studies and provide the most epidemiologically robust information on large numbers of infants. The main limitation of population studies is due to the fact that they often involve multiple observers reporting ROP. This can lead to difficulties of consistent diagnosis and classification.

Most papers published on the incidence of ROP have observed the trends in either a single hospital or a group of hospitals. These findings can be difficult to relate to the population as a whole due to local regional differences in survival rates, neonatal management and ethnicity [Darlow *et al*, 2005; Simpson *et al*, 2003; Vohr *et al*, 2004; Vyas *et al*, 2000]. Darlow *et al* reported the incidence of ROP from 25 NICUs in Australia and New Zealand and found considerable variation in the rates of severe ROP (0-25%) and this remained despite adjustment for case mix and sampling

variability [Darlow *et al*, 2005]. Vohr *et al* found striking differences in neonatal outcomes in America which remained after adjustment for demographics and antenatal interventions, thus suggesting that differences in neonatal care between centres strongly influences outcome neonatal outcome [Vohr *et al*, 2004].

Despite this however, large hospital based cohort studies still provide useful information. Rowlands *et al* found a significant reduction in the incidence of severe ROP in screened infants (BW<1500g or GA<31wks) from 5.7% in 1989 to 1.5% in 1997 in a level 2 neonatal unit in London [Rowlands *et al*, 2001]. Hussain *et al* reported on a similar time period (1989-1997) from a level 3 neonatal unit in the USA and found an incidence of 5% for severe ROP [Hussain *et al*, 1999]. O'Connor *et* al found no significant trend in the incidence of threshold ROP (2-5%) in a single American centre between 1994 and 2000 [O'Connor *et al*, 2003] as did Larsson *et al* in Sweden (severe ROP 18.2% versus 20%) when comparing the incidence of severe ROP in 1988-1990 with 1998-2000 [Larsson *et al*, 2002]. Following adjustment for case mix and sampling variability, Darlow *et al* reported a ≤5.9% incidence of severe ROP in 20% of NICUs within the Australian and New Zealand Neonatal Network in 1998 and 1999 [Darlow *et al*, 2005]. These incidences of severe ROP are similar to our findings and may reflect similar neonatal practices. However, other centres report increases in incidence of severe ROP. Schiariti *et al* reported a marked increase in severe ROP from 7% in 1992-1996 to 14% in 1997-2001 in a tertiary referral hospital in Vancouver, Canada [Schiariti *et al*, 2008]. Vyas *et al* found a significantly higher incidence of severe ROP in one of five English cities studied in 1994 [Vyas *et al*, 2000]. This city also had a lower death rate and so the authors propose that improved survival and development of severe ROP are associated. This study was carried out in very multicultural cities in the Midlands and ethnicity was not accounted for. This may have influenced these results.

In the last decade there have been a number of publications highlighting the role of oxygen free radicals in several neonatal disease processes, as well as ROP [Sola *et al*, 2007; Deulofeut *et al*, 2006; Tin *et al*, 2001]. As a result, many neonatal units have implemented new oxygen saturation policies to reduce the amount of

supplemental oxygen given to premature babies. Several large centres have reported a significant decrease in the incidence of severe ROP following introduction of lower oxygen saturation targets [Vanderveen *et al*, 2006; Wallace *et al*, 2007; Wright *et al*, 2006; Cole *et al*, 2003; Silverman, 2004; Askie *et al*, 2003; Tokuhiro *et al*, 2009; Sears *et al*, 2009]. We did not change our oxygen saturation policy throughout the period of the study. Importantly though, a large randomized trial comparing target oxygen saturations of 85-89% or 91-95% in preterm infants from 24 to 28 weeks gestation yielded a worrying result. There was an increase in mortality (death before discharge) in the lower-oxygen-saturation group [Carlo *et al*, 2010]. The Benefits Of Oxygen Saturation Targeting (BOOST-II) UK trial will provide valuable results on target oxygen saturations and incidence of ROP that should help guide evidence-based neonatal care and help lower the incidence of severe ROP [National Perinatal Epidemiology Unit, 2009].

2.6 Summary

In summary, we have seen an increase in survival of preterm infants, a reduction in the incidence of any degree of ROP, severe ROP and a marked reduction in treatment for ROP in Lothian from 1990-2004. The last 20 years in the developed world have seen dramatic advances in obstetric and neonatal care with routine administration of antenatal corticosteroids for premature labour, use of surfactant therapy, new methods of neonatal mechanical ventilation, introduction of continuous pulse oximetry, use of computerized monitoring systems and advances in neonatal nutritional support. There has also been an increase in centralisation of neonatal care. It is highly probable that these advances are responsible for the increased survival of premature infants and the overall decreasing incidence of ROP seen.

CHAPTER 3

RETINOPATHY OF PREMATURITY IN SMALL-FOR-GESTATIONAL AGE INFANTS

Dhaliwal CA, Fleck BW, Wright E, Graham C, McIntosh N.
Retinopathy of prematurity in small-for-gestational age infants compared with those of appropriate size for gestational age.
Arch Dis Child Fetal Neonatal Ed. 2009;**94**(3):F193-5.

3.1 Introduction

The aetiology of retinopathy of prematurity (ROP) is multifactorial [Romagnoli, 2009]. There is an increasing body of evidence highlighting the importance of rate of body growth both *in utero* and postnatally in the pathogenesis of ROP [Allegaert *et al*, 2003; Wallace *et al*, 2000; Hellstrom *et al*, 2009]. In early pregnancy, fetal growth is due primarily to an increase in cell number and most fetal weight gain occurs during the second and third trimesters [Yu and Upadhyay, 2004]. There are many reasons why infants are born small-for-gestational age (SGA) but in the western world the majority have been exposed to placental insufficiency during the third trimester leading to growth failure. Premature infants who are born SGA are a vulnerable population for the duration of their life. SGA infants have a greater than average risk of morbidity and mortality from many neonatal disorders [Bardin *et al*, 1997; Regev and Reichman, 2003; Gortner *et al*, 1999]. They are also more likely to exhibit neurodevelopmental deficits in childhood and are at increased risk of developing cardiovascular disease and type 2 diabetes in later life [Sung *et al*, 1993; Low *et al*, 1992; Barker, 1990; Hales *et al*, 1991]. Several studies have indicated that SGA infants are at greater risk of developing ROP and one study has shown that IUGR infants had abnormal retinal vascular morphology in adult life [Bardin *et al*, 1997; Wallace *et al*, 2000; Hellstrom *et al*, 2004].

3.2 Aim

To compare the incidence of ROP in SGA and appropriate-for-gestational age (AGA) infants who underwent eye screening within the Lothian region of South East Scotland over a 15-year period from 1990-2004.

3.3 Methods

3.3.1 Study population

This was a prospective observational cohort study. The study population consisted of all infants who underwent eye screening in Lothian hospitals from January 1st 1990-December 31st 2004. These were infants with GA <32 weeks and/or BW <1500 grams who were screened at one of three hospitals (The Simpsons Memorial

Maternity Pavilion, Edinburgh Royal Infirmary; The Eastern General Hospital, Edinburgh; St. John's Hospital, Livingston).

3.3.2 Eye examination schedule

During the study period, two dedicated paediatric ophthalmologists (Dr Brian W. Fleck and Dr Elizabeth Wright) carried out all the eye screening in Lothian. The eye examination schedule is the same as that detailed in Chapter 2 (2.3.3 Eye Examination Schedule).

3.3.3 Eye examination technique

The eye examination technique is the same as that detailed in Chapter 2 (2.3.4 Eye Examination Technique).

3.3.4 Data collection

Information on all babies whose eyes were screened in Lothian during the study period was stored on a database. For every baby, the date of birth, sex, GA, BW and maximum severity of ROP (stage 1-5) in either eye was stored.

3.3.5 Data analysis

The database was accessed and for every baby the BW was plotted on a sex-specific growth chart {Cooney, K. Growth and developmental record (boys: preterm-12 months; girls: preterm-12 months). 1995. Castlemead Publications}. If the BW was below the 10^{th} percentile for gestational age, the infant was defined as being SGA. If the BW was equal to or greater than the 10^{th} percentile for gestational age, the infant was defined as being AGA. The incidence of ROP in SGA babies was compared to the incidence of ROP in AGA babies in gestational age categories. Statistical analysis was performed using SPSS and the GraphPad Instat programme (GraphPad Software, California, USA). As birth weights and gestational ages did not follow a normal distribution, Mann Whitney tests were used to compare the baseline characteristics of the SGA and AGA babies. Contingency tables were analysed in gestational age categories using Chi-square test with Yate's continuity correction. Fishers Exact test was used where numbers were small (<5 infants). In all cases, a two-sided p-value of <0.05 was taken to indicate statistical significance.

3.4 Results

3.4.1 Study population

1668 infants were registered for eye screening during the study period. 168/1668 (10%) were transferred to a neonatal unit outwith Lothian prior to eye screening, 77/1668 (5%) were discharged home prior to eye screening or failed to attend outpatient eye screening and there were insufficient medical records from 10 (<1%). Thus complete data was available from 1413 infants. 329/1413 (23%) of the study population were SGA. The baseline characteristics are shown in table 3.1.

Table 3.1: Baseline characteristics of study population.

	Number of infants (male:female)	Median BW grams (interquartile range)	Median GA weeks (interquartile range)	Number of infants GA>/=32weeks
AGA infants	1084 (617:438)*	890 (585-1170)	27 (26-31)	25
SGA infants	329 (130:194)#	1035 (825-1235)	31 (29-32)	125

* sex not recorded for 29 babies

sex not recorded for 5 babies

The SGA infants had a higher median birth weight and gestational age than the AGA infants (Mann Whitney $p<0.0001$ for both).

3.4.2 Prevalence and severity of ROP.

The maximum severity of ROP recorded for every study baby is presented in Table 3.2. One AGA baby (GA 24 weeks) in 1999 developed stage 4 ROP after laser treatment. No babies developed stage 5 ROP during the study period. The numbers of AGA and SGA infants with any stage of ROP (1-5), severe (stage 3 or greater) ROP and treated ROP were compared using Chi-square test or Fishers exact test where cell numbers were small. The findings are displayed in table 3.2. SGA infants born at gestational ages 26-31 weeks were more likely to develop any stage of ROP ($p<0.01$) than their AGA peers. They were also more likely to develop severe ROP (GA 26-27wks $p<0.01$, GA 28-29wks $p=0.01$, GA 30-31wks $p=0.01$).

Table 3.2: Maximum severity of ROP (stages 1-5) in AGA and SGA infants.

	</=25 wks	26-27 wks	28-29 wks	30-31 wks	>/=32 wks
n-Without ROP (AGA:SGA) [%AGA;%SGA]	27:0 [2:0]	112:6 [10:2]	265:51 [24:16]	429:83 [40:25]	25:123 [2:37]
n-Stages 1,2 ROP (AGA:SGA) [%AGA;%SGA]	31:3 [3:1]	39:10 [4:3]	25:10 [2:3]	15:11 [1:3]	0:1 [0:<1]
n-Stage 3 untreated (AGA:SGA) [%AGA;%SGA]	19:0 [2:0]	12:11 [1:3]	4:5 [<1:2]	0:1 [0:<1]	0:1 [0:<1]
n-Stage 3 treated (AGA:SGA) [%AGA;%SGA]	51:3 [5:1]	28:8 [3:2]	2:1 [<1:<1]	0:1 [0:<1]	0:0 [0:0]
p value* of difference: any stage of ROP	0.46	**<0.01**	**<0.01**	**<0.01**	1.00^
p value* of difference: severe (stage 3-5) ROP	1.00^	**<0.01**	**0.01**	**0.03^**	1.00^
p value* of difference: treated ROP	1.00^	0.59	1.00^	0.46^	-----

* p values relate to comparison of AGA and SGA infants using chi square test;
p values not corrected for the multiple comparisons

^-Fishers exact test used due to small numbers

3.5 Discussion

In our study population, SGA infants born at gestational ages 26-31 weeks were more likely to develop any stage of ROP and severe ROP than their AGA peers. We did not find this association to be statistically significant in infants with GA</=25 weeks. This may have been because of our small numbers at this gestation but also because these infants are at a high risk of ROP irrespective of whether they are SGA or AGA. We also did not find a statistically significant difference in ROP prevalence in SGA and AGA infants born with GA>32wks and this is almost certainly due to the low risk of developing ROP at this gestational age. When comparing the numbers of SGA and AGA infants requiring treatment for severe ROP, SGA infants were not more likely to require laser therapy. This finding is again most likely to be explained by our small numbers with treated ROP. Interestingly, 23% of our study population were SGA and by the terminology definition, this ought to have been 10%. This high

incidence was observed as we had disproportionately large numbers of more mature babies (GA>32 weeks) but whose birth weights were less than the 10th percentile.

The main strength of our study is that our findings are based on a large cohort of babies over a long period of time with two consistent observers and methodical documentation of eye examination findings. The methods of ophthalmoscopic examination for ROP and the classification system used were the same over the whole study period. The two specialised ophthalmologists have worked together using BIO to perform all the ROP eye screening examinations in the Lothian region since 1990. In 2007, for the purpose of another study, the examiners undertook inter-observer agreement studies by independently and blindly grading 80 clinical ROP screening RetCam images. They demonstrated 95% agreement on the presence or absence of 'plus' disease, 94% agreement on stage of ROP and 97% agreement on zone of ROP. We are therefore confident that inter-observer variation should be minimal. There may have been some minor variation in neonatal care between the three Lothian hospitals. However, there were no changes in target oxygen saturation policies during the study period. Ethnicity was not formally recorded but the 2001 census in Scotland reported the population as being 98% Caucasian, 1.4% Asian and 0.6% Afro-Caribbean and other ethnic background.

SGA infants are a vulnerable population at higher risk of perinatal morbidity and mortality than their appropriately sized peers. Other studies have also reported on the incidence of ROP in SGA and AGA infants with similar findings to ours. Gortner *et al* reported the incidence of ROP in SGA infants to be more than double that in AGA infants (37% vs. 15%) although there was no difference in prevalence of stage 3 disease between the two groups [Gortner *et al*, 1999]. Bardin *et al* compared two historical cohorts of SGA and AGA infants born between 24-26 weeks gestation. Like us, they found SGA infants to be at increased risk of developing any stage of ROP (90% vs. 58%) and severe ROP (65% vs. 12%) [Bardin *et al*, 1997]. Allegaert *et al* demonstrated that SGA infants were 3.7 times more likely to develop threshold ROP than their AGA peers [Allegaert *et al*, 2003]. They went on to document perinatal growth and found that even when growth restricted (birth weight<25th

percentile) infants displayed normal postnatal growth (i.e. same g/kg/d as AGA infants) they still have a higher risk of developing threshold ROP. Misra *et al* report that SGA infants in their cohort were at increased risk of developing severe ROP earlier than their AGA counterparts [Misra *et al*, 2008].

There are many possible explanations for why SGA infants have an increased incidence of ROP. The majority of SGA infants are small as a result of IUGR. IUGR infants are exposed to changes in organ development due to fetal hypoxaemia, antioxidant deficiency, free oxygen radicals, nutrient restriction and an altered endocrine environment [Regev and Reichman, 2004; Kelly, 1993; Saugstad, 2001]. These developmental changes may be linked to the increase in ROP. SGA infants are often sicker infants than their AGA peers, requiring more intensive and more prolonged hospital care [Yu and Upadhyay, 2004]. Thus their comorbidities may be linked to the increase in incidence seen. SGA infants have lower serum levels of insulin-like growth factor-1 (IGF-1) and there is evidence for the role of this growth factor and vascular endothelial growth factor (VEGF) in the pathogenesis of ROP [Smith, 2005]. Certainly there is scope to study prenatal and postnatal growth rates in premature infants in relation to later development of ROP as there is great potential to modify nutrition which may ameliorate ROP development.

Early nutrition has a 'programming' effect on health in early adulthood, notably on cardiovascular disease risk, bone health and cognitive function [Lucas, 2005]. It is possible that a stimulus such as placental insufficiency during the third trimester may 'program' the fetus to develop vascular disease in the neonatal period such as ROP.

3.6 Summary

In our study population we found that the prevalence of ROP was higher in SGA infants than AGA infants. In addition, the prevalence of severe (stages 3-5) disease was greater in SGA infants. There are many possible explanations for this observed association and I was keen to explore the role of nutrition and growth further. This led me to adapt the 'Edinburgh rat model of ROP' in order to study the association

between growth and retinal vascular development. This is explained in the following chapter.

CHAPTER 4

THE INFLUENCE OF GROWTH, INSULIN-LIKE GROWTH FACTOR-1 AND OXYGEN ON RETINAL VASCULAR DEVELOPMENT:- DEVELOPMENT OF AN ANIMAL MODEL

4.1 Introduction

Normal fetal growth is a complex process dependent on the genetic profile of the embryo, adequate nutrient and oxygen supply via the placenta and the fetal-maternal hormonal and growth factor milieu. Insulin-like growth factors are key growth factors in fetal growth and development [van Kleffens *et al*, 1998]. This process is interrupted following preterm birth and premature infants are therefore born with incompletely vascularised retinas which must complete development in the postnatal period. Retinopathy of prematurity (ROP) develops as a result of abnormal postnatal retinal vascular development.

Premature infants who are born small-for-gestational age (SGA) are at increased risk of developing retinopathy of prematurity (ROP) [Dhaliwal *et al*, 2008; Bardin *et al*, 1997; Darlow *et al*, 2005]. Premature infants who suffer from postnatal growth retardation are also at increased risk of developing ROP [Wallace *et al*, 2000; Lofqvist *et al*, 2006; Hellstrom *et al*, 2009]. These facts suggest that the nutritional status of the fetus and premature neonate may influence retinal vascular development.

Animal models are useful tools to try and understand the mechanisms behind observed clinical associations. The rat is a suitable model in which to study retinal vascularisation as this occurs in the first 14 days after birth [Henkind, 1967]. The Edinburgh Rat Model of ROP exposes rat pups to a fluctuating oxygen profile from birth to day 14 of life [Cunningham *et al*, 2000]. This model induces rat retinal changes similar to those observed in clinical ROP.

We adapted the Edinburgh Rat Model of ROP in order to study the effect of growth restriction on rat retinal development. We also wanted to study insulin-like growth factor-1 (IGF-1) in this model as there is evidence to suggest that it may play a key role in the observed association between growth restriction and ROP [Smith, 2005].

4.2 Methods

This study was approved by the UK Home Office and all animals were cared for in accordance with UK Home Office legislation.

4.2.1 The Edinburgh Retinopathy of Prematurity oxygen chamber (figure 4.1)

This animal model of ROP was developed in Edinburgh [Cunningham *et al*, 2000]. Edinburgh Neonatal Intensive Care Unit has stored computerized data on arterial oxygen concentrations of infants. Arterial oxygen concentrations were measured transcutaneously and a mean of data points for every minute were stored. The minute-to-minute oxygen profile over the first 14 days of life from one infant who developed threshold ROP was taken. The infant's arterial oxygen profile was converted into an equivalent oxygen profile for the rat. A computer-controlled system delivered this oxygen profile to the rat litters by releasing oxygen and nitrogen gases on a minute-by-minute basis into a closed animal chamber (BioSpherix Ltd, New York). Changes in retinal vascularisation on the rat retina were seen that were similar to those observed in human infants with ROP. Rat pups raised in this variable oxygen profile around a hyperoxic mean inspired oxygen concentration have been shown to have more severe retinal vascular abnormalities [McColm *et al*, 2004]. For this work, rats were exposed to the mildly hyperoxic variable oxygen profile about a mean inspired oxygen concentration of 14.9kPa (24.7% FiO_2) (equivalent to neonatal arterial oxygen concentration of 10kpa).

4.2.2 Animals

Pregnant Sprague-Dawley rats were used. From day 15 gestation, they were fed either an isocaloric low protein (9% casein) diet (to induce growth restriction) or a 'normal protein' (18% casein) diet (see appendix section 1.3). The same diet was continued following the birth of pups. Water was available *ad libitum*. Dam and pups were weighed on the day of pup birth. Litter size was standardised to 10-12 pups. Two experimental groups and two control groups of animals were studied (figure 4.2).

Figure 4.1: The Edinburgh Rat Model of ROP

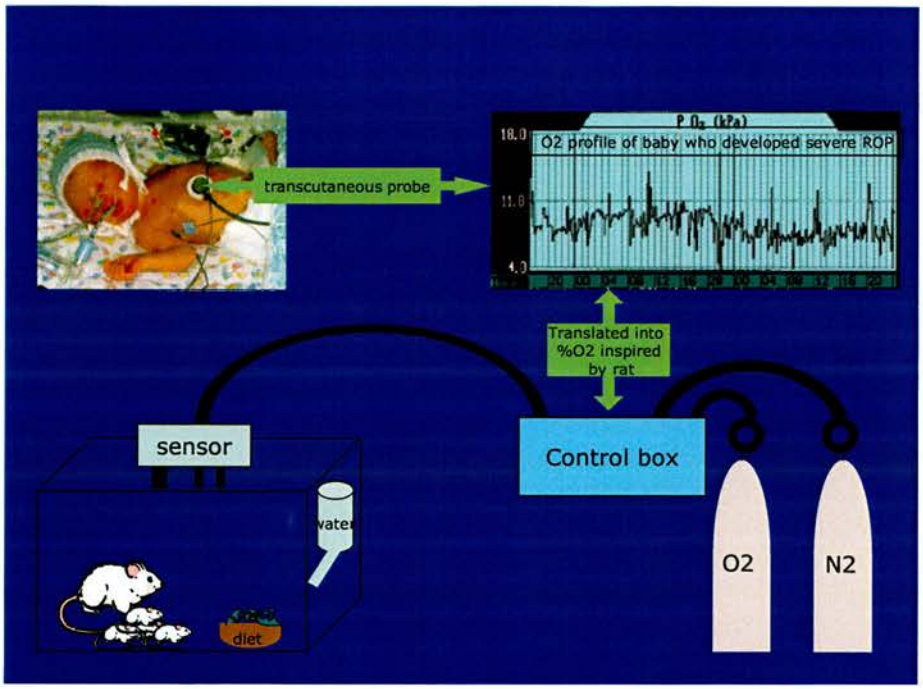
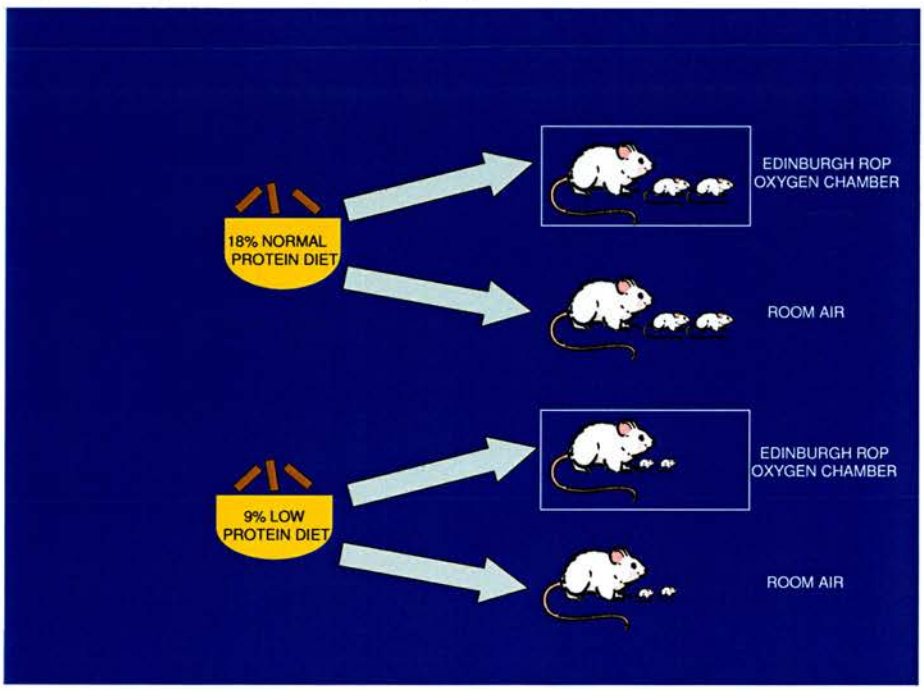


Figure 4.2: Experimental and control groups of animals



On the day of birth, some litters were placed in the oxygen chamber and the variable oxygen profile commenced whilst other litters remained in cages in room air. Soda lime was placed in the enclosed oxygen chamber in order to maintain the carbon

dioxide concentration at normal atmospheric levels. Light was cycled on a 12 hour on, 12 hour off schedule and the room temperature was maintained at approximately 21 degrees centigrade. The variable oxygen profile was paused briefly on day 7 in order to change bedding. Bedding was also changed for room air litters on day 7. Experiments were terminated on day 14.

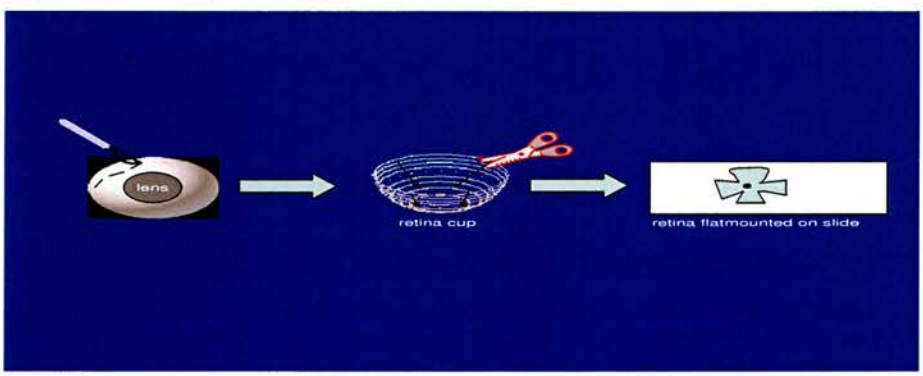
4.2.3 Tissue collection

On day 14, dam and pups were weighed. Pups were anaesthetised by intraperitoneal injection of ketamine (2.5mg/kg) and xylazine (1mg/kg). The thoracic cavity was opened. A blood sample was taken from the left ventricle using 25 gauge needle and 1ml syringe. A cut was then made in the liver (to permit exsanguination) and 10mls of phosphate-buffered saline (PBS-see appendix section 1.2) pH 7.4, followed by 5mls of 0.5% paraformaldehyde (PFA, Sigma-Aldrich) was injected into the left ventricle of the heart. These fluids flushed blood out and initiated tissue fixation. Pups were then killed by intracardiac injection of pentobarbitone (80mg/kg). Both eyes were enucleated.

4.2.4 Tissue processing-retinas (Figure 4.3)

Enucleated eyes were immediately fixed in 4% PFA for one hour and then washed in PBS. The cornea, lens and vitreous of the eyes were surgically removed and the retinas were dissected using the method described by Chan-Ling [Chan-Ling, 1997]. Four incisions were made in the 'cup-shaped' retinas and they were wholemounted on TESPA (3'-aminopropyltriethoxysilane) coated microscope slides (figure 4.3). A rectangle was drawn on the slide around each retina using a Dako pen [Dako UK Ltd] in order to contain the solutions applied during processing.

Figure 4.3: Tissue processing-retina



The immunohistochemical staining regime described below was designed in order to label both the retinal astrocyte cells and retinal endothelial cells. My colleague (JW) studied the astrocytes (which I shall not discuss) and I studied the endothelial cells.

The retinas were fixed in 70% ethanol (stored at -20°C) for 20 minutes with gentle shaking and then washed three times in PX (see appendix section 1.2) for 5 minutes each time. Blocking buffer (see appendix section 1.2) was applied for 2 hours at room temperature. The primary antibody for astrocyte staining (rabbit anti-cow GFAP, diluted 1 in 1000 in blocking buffer [Dako UK Ltd]) was applied and retinas were incubated overnight at 4^0C. Retinas were washed three times in PX (5 minutes per wash). The secondary antibody for astrocyte staining (swine anti-rabbit IgG biotinylated, diluted 1 in 200 in PX [Dako UK Ltd]) was added for 2 hours at room temperature with gentle shaking. Retinas were again washed three times in PX (5 minutes per wash). The fluorescent label for astrocyte staining (streptavidin alexa fluor 568, red, diluted 1 in 200 in PX [Invitrogen Ltd UK]) was applied and retinas incubated for 2 hours at room temperature with gentle shaking in the dark. The retinas remained in the dark for the subsequent immunohistochemical stages. Retinas were then washed three times in PX (5 minutes per wash). In order to block non-specific binding during the endothelial cell staining, the retinas were first incubated with avidin for 20 minutes and then biotin for 20 minutes at room temperature [avidin/biotin blocking kit, Vector Laboratories Ltd, UK]. Retinas were incubated with biotinylated lectin (*Griffonia simplicifolia* Bandeiraea isolectin IB4, diluted 1 in 40 in PX [ICN Pharmaceuticals Ltd, UK]) overnight at 4°C to label the endothelial cells. The retinas were washed three times in PX (5 minutes per wash) before incubating with the endothelial cell fluorescent label (fluorescein streptavidin, green, diluted 1 in 100 in PX [Sigma-Aldrich, UK]) for 2 hours at room temperature with gentle shaking. Slides were washed three times in PX (5 minutes per wash) and once in PBS for 5 minutes and mounted using mowiol (see appendix section 1.2).

4.2.5 Imaging, image analysis and statistics

Images of the stained flatmounted retinas were taken using an argon krypton laser confocal microscope (Leica TCS-NT, Leica Microsystems, Bucks, UK). Digitalised

whole retina images were reconstructed using Adobe Photoshop (Adobe Systems Incorporated) and stored as jpeg files (figures 4.4, 4.5).

Figure 4.4: sample retina from 14 day rat pup (room air, 18% diet)

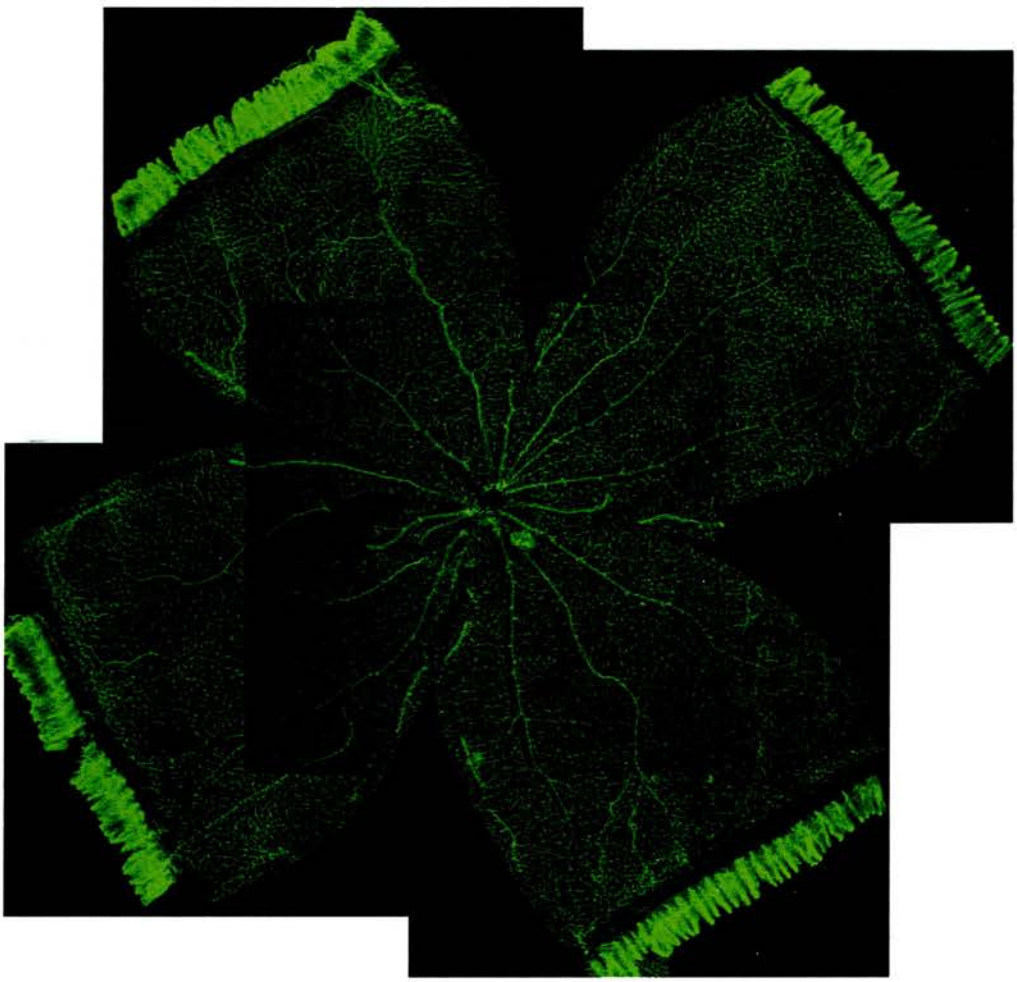
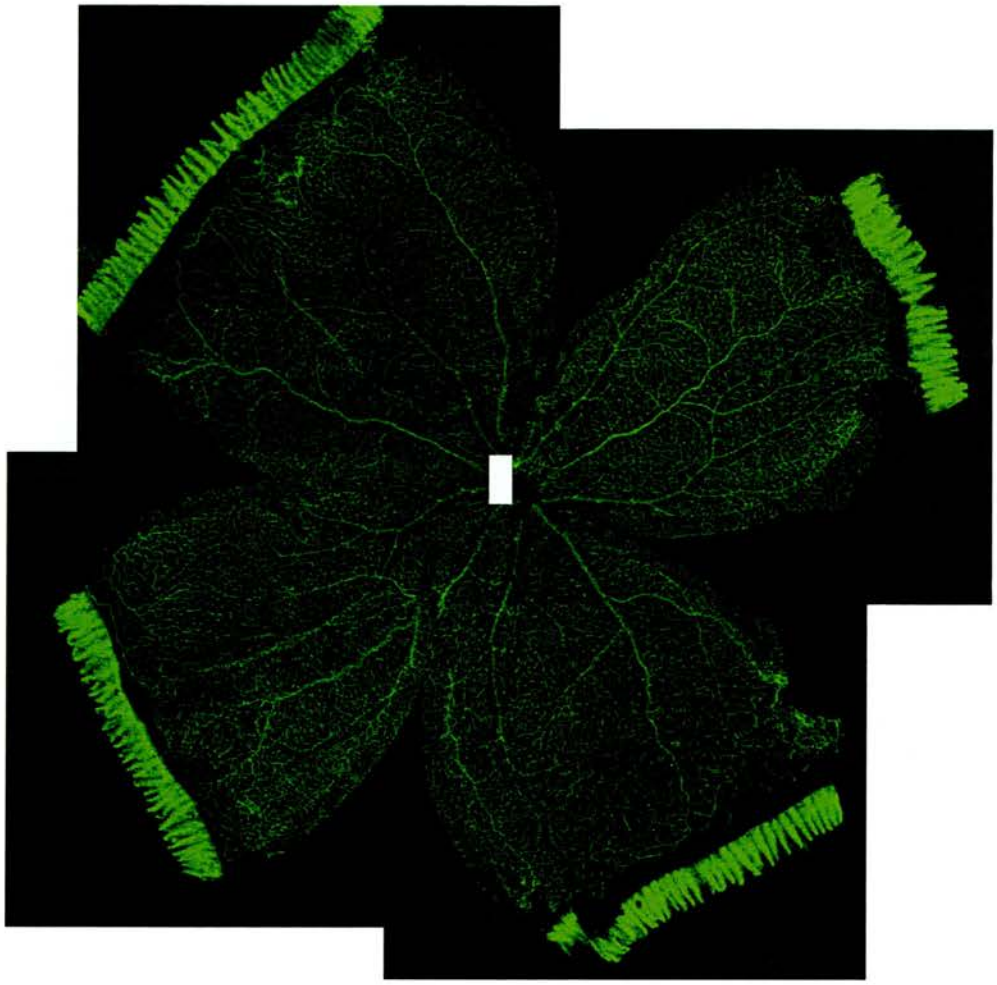


Figure 4.5: sample retina from 14 day rat pup (room air, 9% diet)



For every retina, the avascular retinal area and total retinal area was measured using Image J Software. The analyser (CD) was blind to the experimental conditions. The avascular area was expressed as a proportion of the total retinal area. A one-way analysis of variance (ANOVA) was carried out to study the effect of independent variables (pup birthweight, pup weight gain, 9% diet, 18% diet, room air, variable oxygen) on the dependent variable (the avascular area). The computer package SPSS was used.

Mean pup birth weights and day 14 weights were calculated and compared using an unpaired t test and Mann Whitney test respectively. Statistical analysis was performed using the GraphPad Instat programme (GraphPad Software, California, USA). In all cases, a p-value of <0.05 was taken to indicate statistical significance.

4.2.6 Serum IGF-1 analysis and statistics

Blood was taken from day 14 rat pups as described above. Blood was also taken from day 7 rat pups which were being used for different research work within our group. These pups were reared under identical experimental conditions. Blood samples were allowed to clot for 2 hours at room temperature. They were then centrifuged for 20 minutes at 1000 x g. Serum was removed and stored at -70°C until assayed.

A quantikine mouse IGF-1 immunoassay kit [R&D Systems Europe Ltd, UK] was used to quantify serum IGF-1. This was an enzyme-linked immunosorbent assay (ELISA) which had also been validated for rat IGF-1. Rat serum samples were diluted 1000-fold for the assay. The kit contained a monoclonal antibody specific for IGF-1 pre-coated onto a microplate. Standards, controls and samples were pipetted into the microplate wells. These were all assayed in duplicate. The immobilised antibody bound to any IGF-1 and any unbound substances were washed away. An enzyme-linked polyclonal antibody specific for IGF-1 was then added to the wells. The wells were washed to remove any unbound substances before a substrate solution was added. The enzyme reaction yielded a colour change that was

terminated by addition of the stop solution. The intensity of the colour was measured using a microplate reader and was in proportion to the amount of rat IGF-1 bound in the first step. The standard, control and sample duplicate readings were averaged and the average zero standard optical density was subtracted. A standard curve was constructed using GraphPad Prism computer software and sample values were read off the standard curve. The values were then multiplied by the dilution factor of 1000 to give serum concentrations measured in ng/ml.

One-way ANOVAs were carried out to study the effect of independent variables (pup weight gain, 9% diet, 18% diet, room air, variable oxygen) on the dependent variable (serum IGF-1) on results from both day 7 and 14 pups. The computer package SPSS was used.

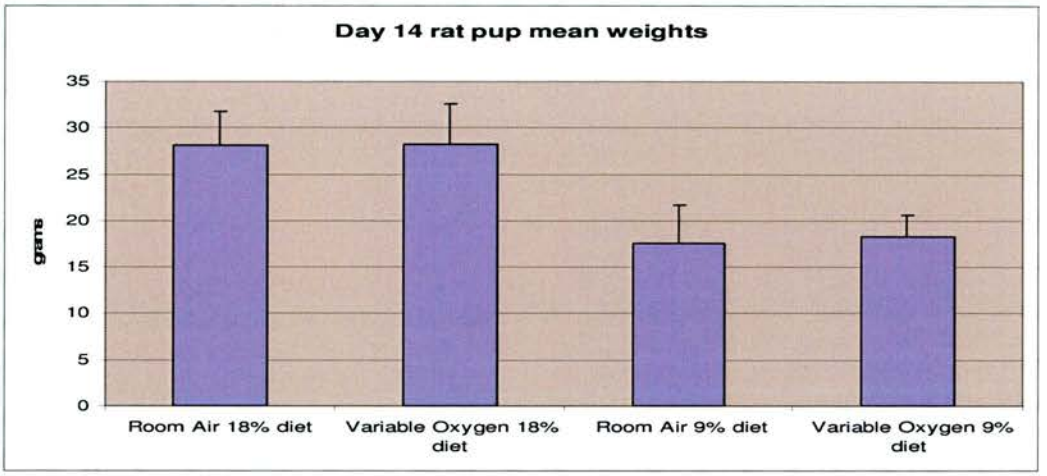
4.3 Results

4.3.1 Pup weights (Figure 4.6)

The mean birth weight of pups from dams fed the 18% normal protein diet was 5.73g (SD 0.70) and mean birth weight of pups from dams fed the 9% low protein diet was 5.46g (SD 0.68){unpaired t test comparing mean birth weights $p<0.0001$}. The pups from dams fed the low protein diet weighed on average 5% lighter at birth.

The mean day 14 weight of pups in the four experimental and control groups is shown in figure 10 {Mann Whitney tests: comparing room air 18% diet with room air 9% diet mean weights $p<0.0001$, comparing variable oxygen 18% diet with variable oxygen 9% diet mean weights $p<0.0001$}. Pups from dams fed the low protein diet weighed on average 36% lighter than those from dams on the normal protein diet at day 14.

Figure 4.6: Day 14 rat pup mean weights {bars=standard deviation}



4.3.2 Retinal avascular areas

The results are shown in table 4.1. Growth restricted (9% protein diet) pups had smaller total retinal areas than 'normal size (18% protein diet) pups as they had smaller eyes.

Table 4.1: Mean retinal areas in four animal groups

	Mean retinal avascular area mm^2 (SD)	**Mean total retinal area mm^2 (SD)**	**Mean [avascular area/total retinal area] mm^2 (SD)**
Normal (18%) protein diet Room Air (n=31 pups from 6 litters)	0.602 (0.154)	36.271 (2.661)	0.017 (0.004)
Normal (18%) protein diet Variable Oxygen (n=23 pups from 4 litters)	0.682 (0.252)	37.330 (1.461)	0.018 (0.007)
Low (9%) protein diet Room Air (n=29 pups from 5 litters)	0.784 (0.267)	33.743 (3.409)	0.023 (0.007)
Low (9%) protein diet Variable Oxygen (n=27 pups from 4 litters)	1.051 (0.299)	34.965 (3.056)	0.038 (0.041)

A credible ANOVA requires that the variances within each group are the same. This was tested for using Levene's test. The initial test showed that the variances were not equal and so the data was transformed using a square root transformation. A repeat Levene's test showed equality of variance (p=0.060) so an ANOVA was done on the transformed data [square root of (avascular area/total retinal area)]. The results are shown in table 4.2.

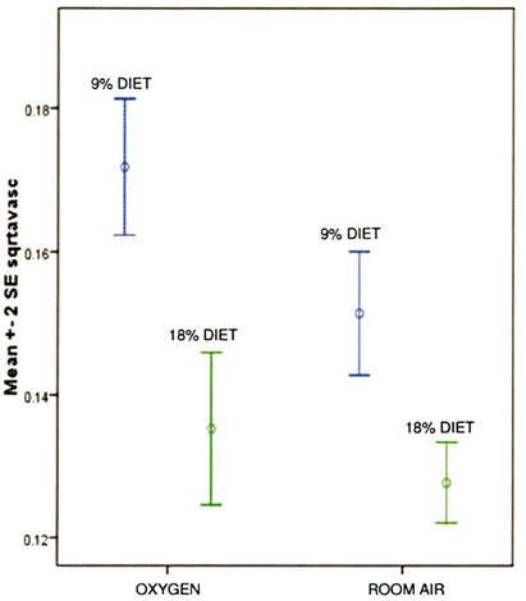
Table 4.2: ANOVA Tests of Between-Subjects Effects [dependent variable: square root (avascular area/total retinal area)]

Source	Type III Sum of Squares	Degrees of freedom	Mean square	F	Significance
Corrected Model	0.33	5	0.007	13.431	**0.000***
Pup birth weight	0.001	1	0.001	2.291	0.133
Pup weight gain$	2.087E-5	1	2.087E-5	0.042	0.838
Oxygen concentration	0.006	1	0.006	12.271	**0.001***
Diet (9% or 18%)	0.004	1	0.004	7.324	**0.008***
Gas/diet interaction	0.000	1	0.000	0.409	0.524
Error	0.051	104	0.000		
Total	2.441	110			
Corrected Total	0.085	109			

$ pup weight gain=(day 14 weight-birth weight)/birth weight

The ANOVA (table 4.2) shows a significant main effect for oxygen concentration (air or oxygen) and diet (9% or 18%). The effect of oxygen concentration (air or oxygen) is more significant than the effect of diet (9% or 18%). These findings are illustrated graphically in figure 4.7. There is no significant interaction effect between gas and diet (see table 4.2).

Figure 4.7: Effect of diet and gas on retinal avascular area



The ANOVA shows no significant independent effect of birth weight or pup weight gain.

4.3.3 Serum IGF-1

The number of serum IGF-1 samples measured for each of the four groups of animals is shown in table 4.3.

Table 4.3: Number of litters and pups used to measure serum IGF-1

	Room air 18% diet	**Variable O_2 18% diet**	**Room air 9% diet**	**Variable O_2 9% diet**
Day 7 serum Number of pups	26	28	29	25
Day 7 serum Number of litters	3	3	3	3
Day 14 serum Number of pups	30	32	29	20
Day 14 serum Number of litters	3	3	3	3

All control samples were within the recommended kit concentration range. Levene's test for homogeneity at both 7 and 14 days was significant and this could not be corrected by a transformation. Results from the one-way ANOVAs at day 7 and 14 are tabulated in tables 4.4 and 4.5 with the Levene p value reported. There are limitations of using ANOVA in this situation but figures 4.8 and 4.9 illustrate clearly why the variances were not equal.

Table 4.4: Day 7 results:-ANOVA Tests of Between-Subject Effects [dependent variable: serum IGF-1] Levene p=0.000

Source	Type IV Sum of Squares	Degrees of freedom	Mean Square	F	Significance
Corrected Model	361967.785	4	90491.946	25.203	**0.000***
Pup weight gain$	37210.331	1	37210.331	10.364	**0.002***
Oxygen concentration	8327.473	1	8327.473	2.310	0.131
Diet (9% or 18%)	50499.016	1	50499.016	14.065	**0.000***
Gas/diet interaction	1203.821	1	1203.821	0.335	0.564
Error	369819.116	103	3590.477		
Total	3914826.570	108			
Corrected Total	731786.901	107			

$ pup weight gain=(day 14 weight-birth weight)/birth weight

Table 4.5: Day 14 results:-ANOVA Tests of Between-Subject Effects [dependent variable: serum IGF-1] Levene p=0.000

Source	Type IV Sum of Squares	Degrees of freedom	Mean Square	F	Significance
Corrected Model	472185.242	4	11	40.393	**0.000***
Pup weight gain$	114993.901	1	114993.901	39.349	**0.000***
Oxygen concentration	1201.671	1	1201.671	0.411	0.523
Diet (9% or 18%)	18420.913	1	18420.913	6.303	**0.014***
Gas/diet interaction	310.572	1	310.572	0.106	0.745
Error	309776.906	106	2922.424		
Total	4053709.091	111			
Corrected Total	781962.147	110			

$ pup weight gain=(day 14 weight-birth weight)/birth weight

The ANOVAs (tables 4.4, 4.5) show a significant main effect for diet (9% or 18%) and pup weight gain at both time points. The effect of oxygen concentration (air or oxygen) is not significant. These findings are illustrated graphically in figures 4,8 and 4.9.

Figure 4.8: Effect of diet and gas on serum IGF-1 at pup day 7 of life

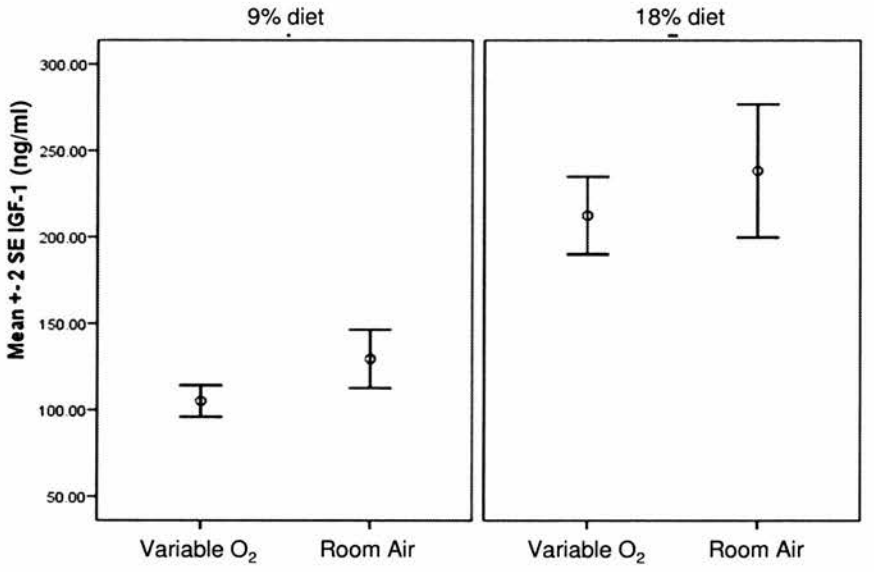
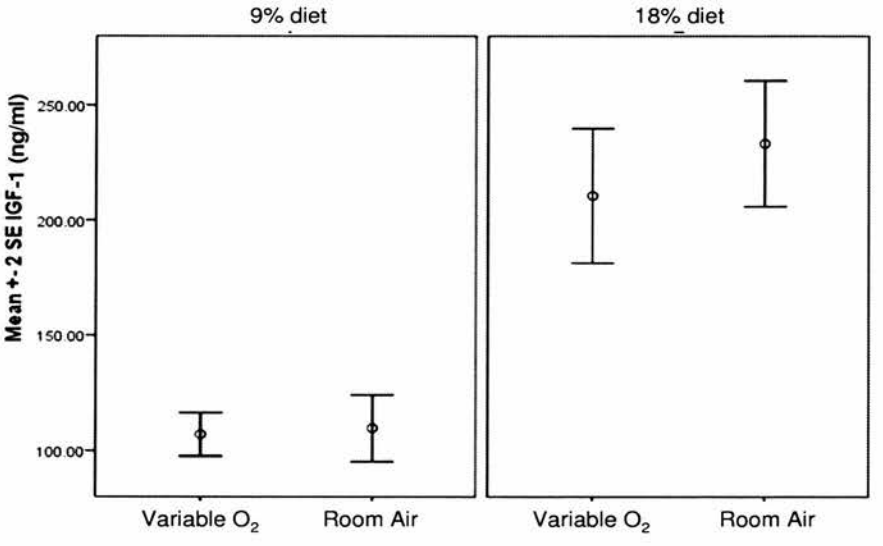


Figure 4.9: Effect of diet and gas on serum IGF-1 at pup day 14 of life



Non-parametric (Mann-Whitney) tests were carried out in order to confirm the differences for the ANOVAs reported in tables 5 and 6 and to help explain why the variances could not be made homogeneous. At both day 7 and day 14, oxygen concentration did not have an effect on serum IGF-1 (p=0.45, p=0.848 respectively) and diet had a significant effect on serum IGF-1 (p=0.000 for both).

4.4 Discussion

We have shown that a 9% low protein diet caused prenatal and postnatal growth restriction in rat pups. Growth restricted rat pups had significantly larger retinal avascular areas than normally grown rat pups (p=0.008) following exposure to both room air and variable oxygen. This means that the area of the retina covered by blood vessels was smaller in growth restricted pups and indicates that retinal vascularisation was delayed. In addition, rat pups exposed to variable oxygen had significantly larger avascular areas than rat pups exposed to room air (p=0.001) on both diets. Growth restricted rat pups had significantly lower serum IGF-1 levels at postnatal days 7 and 14 than normally grown pups (ANOVA test effect of diet on IGF-1 p=0.000 day 7, p=0.014 day 14). Serum IGF-1 levels were associated with pup weight gain (p=0.002 day7, p=0.000 day 14) meaning that pups gaining weight more slowly had lower serum IGF-1 levels. Exposure to air or variable oxygen did not influence serum IGF-1 levels.

The main advantage of the Edinburgh rat model of ROP is that it is physiological. We did not observe neovascularisation in our model but we did observe significant changes in the size of the avascular retinal areas. This is an important early stage in retinal vascularisation because the longer the retina remains avascular, the more it is at risk of abnormal neovascularistion. We therefore believe our model to be an important model in which to study early retinal vascular changes that precede frank neovascularistion characteristic of severe ROP. The model has previously shown that pups exposed to the variable oxygen profile have larger retinal avascular areas and our findings support this but demonstrate further that the largest avascular areas are seen in growth restricted rat pups raised in variable oxygen. Growth restriction together with variable oxygen hinders retinal vascularisation. The main disadvantage

of our *in vivo* model is that it does not cause frank neovascularisation that is seen in humans and other animal models of ROP [Smith *et al*, 1994; Penn *et al*, 1994]. Cunningham *et al* reported that the Edinburgh model caused development of 'neovascular tufts' but we were not able to visualise these in our experiments [Cunningham *et al*, 2000]. One possible explanation for why frank neovascularisation was not observed is due to rat strain differences. The incidence of neovascularisation in oxygen exposed rats has been shown to be much higher in Brown Norway rather than Sprague Dawley strains [Floyd *et al*, 2005].

Several animal models of intrauterine growth restriction (IUGR) have been developed. These include nutritional manipulation, surgical uterine artery ligation, hypoxia and drug induced methods [Holemans *et al*, 1998; Snoeck *et al*, 1990; Barker, 1990; Woodall *et al*, 1996; Wigglesworth, 1964; de Grauw *et al*, 1986; Benediktsson *et al*, 1993; Langley-Evans *et al*, 1994; Hohmann *et al*, 1992]. We chose to give the low protein diet from day 15 gestation as this has been shown to cause late gestation impairment of truncal growth with little effect on brain growth [Langley-Evans and Nwagwu, 1998]. IUGR in humans is most commonly asymmetrical, occurring in the last trimester as a result of a failing placenta. Premature infants have an increased metabolic rate and have a high protein loss in the early couple of days of life [Denne, 2001]. The diet of preterm infants in intensive care is also high in fat and carbohydrate and low in protein [Vasu and Modi, 2007]. We therefore felt that this model most closely mimicked the human clinical situation. The rat low protein nutritional model causes reduced substrate delivery to the fetoplacental unit, decreased uteroplacental blood flow and reduction in total milk volume during lactation. These all hamper neonatal rat pup growth [Holemans *et al*, 1998].

We have shown that growth restricted rat pups had larger avascular areas following exposure to both room air and variable oxygen than normally grown pups. We have also shown that growth restricted rat pups had significantly lower levels of serum IGF-1 at postnatal days 7 and 14. These associations reflect the clinical observations in human preterm infants that slow postnatal growth is associated with low serum

IGF-1 levels and an increased risk of developing ROP [Lofqvist *et al*, 2006; Wallace *et al*, 2000; Allegaert *et al*, 2003]. Unfortunately we were not able to relate individual rat pup retinas to the corresponding pup serum IGF-1. This was due to technical difficulties in retinal dissection, staining and in some cases low volume of serum obtained. Nutrition is a key regulator of IGF-1 production in both pre- and postnatal life [Davenport *et al*, 1990]. It has previously been shown that restriction in protein intake in humans and animals leads to a significant reduction in serum concentration of IGF-1 and IUGR [Woodall *et al*, 1996]. Our findings support this and we postulate that serum IGF-1 concentration may have a direct effect on retinal vascularisation with a low serum IGF-1 being associated with slower retinal vascular development.

Vanhaesebrouck *et al* carried out a very similar set of experiments to ours except they studied the effect of postnatal rat pup growth restriction [Vanhaesebrouck *et al*, 2009]. They raised pups in small or large litters and measured body weight, serum IGF-1, retinal avascular area and number of neovascular nuclei. They found that in smaller litters, mice were heavier with higher serum IGF-1 levels and less neovascularisation. Like us they found that body weight influenced serum IGF-1. They did however also find that IGF-1 strongly correlated with the percentage avascular area ($r=0.53$; $p<0.0001$) and with the number of neovascular nuclei ($r=0.53$; $p<0.0001$). This supports our theory that serum IGF-1 levels have a direct effect on normal retinal blood vessel development. Vanhaesebrouck *et al* also gave the mice a single dose of exogenous IGF-1 and this had a profound effect by improving body weight, increasing endogenous IGF-1 levels and reducing neovascularisation ($p=0.00001$) [Vanhaesebrouck *et al*, 2009]. Holmes *et al* also studied the effect of postnatal growth restriction (induced by raising pups in large litters) on retinal vascularisation and found that growth retarded pups developed more severe abnormal retinal neovascularisation [Holmes *et al*, 1996].

There is now a wealth of data supporting the importance of IGF-1 in normal and abnormal retinal vascularisation but the mechanisms behind how IGF-1 affects retinal vascularisation are still being described [Hellstrom *et al*, 2003; Hellstrom *et*

al, 2001; Hellstrom *et al*, 2004; Lofqvist *et al*, 2006]. Hypoxia-inducible factor-1α (HIF-1α) is a key oxygen dependent transcription factor but can also be activated in an oxygen-independent way. IGF-1 has been shown to act though a posttranscriptional mechanism to cause HIF-1α accumulation, nuclear translocation and increased activity [Treins *et al*, 2005]. IGF-1 stimulates HIF-1α activity through signalling pathways that lead to vascular endothelial growth factor (VEGF) mRNA expression. It appears likely that hypoxia and IGF-1 lead to HIF-1α activation and downstream VEGF expression by independent signalling pathways which act together to cause the neovascularisation seen in ROP [Treins *et al*, 2005]. IGF-1 has also been shown to have a direct effect on VEGF. A low level of IGF-1 has been shown to prevent the *in vitro* activation of VEGF-induced protein kinase B (Akt), which is a critical kinase involved in endothelial cell survival [Hellstrom *et al*, 2001]. It remains likely however that the fine balance of normal retinal blood vessel development versus frank neovascularisation is a sophisticated interplay of multiple components and pathways although the effects of IGF-1 on HIF-1α and VEGF appear to be key interactions.

Although unlikely, it also remains possible that our observed larger avascular areas in the growth restricted pups may not be related to serum IGF-1 levels at all. It is possible that the serum IGF-1 levels are just a reflection of body weight and have no interaction with retinal blood vessel development. Other possible mediators for the observed association between growth restriction and blood vessel development are the glucocorticoids. Feeding of a low protein diet during pregnancy in the rat is associated with increased fetal glucocorticoid exposure and effects on the structure and function of the endocrine pancreas in the pups [Holemans *et al*, 1998; Snoeck *et al*, 1990; Langley-Evans and Nwagwu, 1998]. Glucocorticoids are powerful biological molecules and have been implicated in the prenatal programming of hypertension and impaired glucose tolerance in later life [Langley-Evans and Nwagwu, 1998]. Their receptors are highly expressed in most fetal tissues and therefore they may also be involved in retinal vascular development [Holemans *et al*, 1998]. Whether they play a role in the pathogenesis of ROP remains poorly understood.

Our animal model has been a useful tool in trying to further understand and describe the association between growth restriction, IGF-1 and ROP. However, necessary caution is required when extrapolating our findings to the clinical situation. It is very likely that rat dam hormonal milieu, placental function and fetal response to growth restriction is different to human response.

4.5 Summary

We have demonstrated that growth restricted rat pups have larger retinal avascular areas than normally grown pups. Rat pups exposed to a variable oxygen profile also have larger retinal avascular areas than pups exposed to room air. Larger retinal avascular areas infer less retinal blood vessel coverage. These findings have important clinical implications and are consistent with our clinical observation that small-for-gestational age infants are more likely to develop severe ROP. In the premature infant, the longer the retina takes to vascularise, the more at risk the infant is of developing ROP.

We also demonstrated that growth restricted rat pups had significantly lower serum IGF-1 levels at postnatal days 7 and 14 than normally grown pups. Serum IGF-1 levels were influenced by pup weight gain. We hypothesise that serum IGF-1 concentration may have a direct effect on retinal vascularisation in our rat model of ROP, with a low serum IGF-1 being associated with slower retinal vascular development.

Further research is required into nutritional therapies which may improve retinal vascularisation in premature infants and reduce the incidence of sight-threatening ROP.

CHAPTER 5

WIDE-FIELD DIGITAL RETINAL IMAGING VERSUS BINOCULAR INDIRECT OPHTHALMOSCOPY FOR RETINOPATHY OF PREMATURITY EYE SCREENING EXAMINATIONS

Dhaliwal C, Wright E, Graham C, McIntosh N, Fleck BW.
Wide-field digital retinal imaging versus binocular indirect ophthalmoscopy for retinopathy of prematurity screening: a two-observer prospective, randomised comparison.
Br J Ophthalmol. 2009;**93**(3):355-9.

5.1 Introduction

The purpose of retinopathy of prematurity (ROP) eye screening examinations is to identify premature infants with potentially sight-threatening disease. Early detection and treatment of these infants with laser photocoagulation has been shown to significantly reduce the incidence of severe visual loss [Early Treatment For Retinopathy Of Prematurity Cooperative Group, 2003]. Current UK guidelines recommend that all infants with birth weight (BW)<1501g and/or gestational age (GA)<32 weeks are screened for ROP [Royal College of Paediatrics and Child Health, 2008].

The conventional gold standard screening technique is binocular indirect ophthalmoscopy (BIO) with eyelid speculum and scleral indentation. This is a technically difficult procedure and must be performed by an experienced ophthalmologist. In the developed world, adequate ophthalmic expertise is often confined to larger regional neonatal units. It is therefore difficult to provide adequate ROP screening to infants at remote centres. Screening with BIO is even more difficult to implement in 'middle income' countries, where there is currently an epidemic of ROP, which is due in part to the paucity of adequately trained specialists to deliver the service [Gilbert, 2008].

Wide field digital retinal imaging (WFDRI) with eyelid speculum is an alternative screening technique. The digital camera system (RetCam II, Clarity Medical Systems, Pleasanton, California) captures images of the retina which are stored for interpretation. With the advent of 'store and forward' telemedicine, this is an attractive screening tool as trained staff can capture retinal images which can be interpreted by a remote ophthalmologist [Chiang *et al*, 2007; Ells *et al*, 2003; Lorenz *et al*, 1999; Roth *et al*, 2001]. The remote ophthalmologist may be based at a regional or national or even international centre. WFDRI is being developed as a tool for future clinical trials and there is therefore a need to validate WFDRI against BIO.

We have carried out a masked, double-observer prospective randomised comparison of WFDRI and BIO in a consecutive series of ROP screening examinations in our neonatal unit.

5.2 Aim

To compare the diagnostic accuracy of WFDRI with the current 'gold standard' of BIO for ROP screening examinations.

5.3 Methods

This was a prospective, randomised, comparative study. The Lothian Research Ethics Committee (LREC) approved the study. All parents gave informed consent.

5.3.1 Study population

Consecutive infants undergoing routine ROP screening at Edinburgh Royal Infirmary Neonatal Unit were eligible for inclusion in the study. At this centre, all infants born with GA<32 weeks and/or BW <1500g have their eyes screened for ROP. Patients were recruited from June 2004 to May 2007.

5.3.2 Examination Schedule

Screening was carried out by two experienced paediatric ophthalmologists (Dr Brian Fleck and Dr Elizabeth Wright). Infants were first examined at a chronological age of 4-6 weeks or corrected age of 34 weeks, whichever was earlier. Screening was continued fortnightly if no ROP was present and weekly if any ROP was seen. Screening continued until the retina was normally vascularised into zone 3, the infant required treatment or until the infant was transferred to an outlying hospital. On each screening occasion, study infants had both eyes examined by both BIO and WFDRI. The study coordinator (Dr Catharine Dhaliwal) randomised both examiners to screening using either BIO or WFDRI and also randomised the order in which the examinations were to be carried out. Randomisation was carried out for each examination using sealed envelopes.

5.3.3 Examination Technique

Pupils were dilated with topical phenylephrine 2.5% and tropicamide 0.5% applied 60 minutes and 30 minutes prior to the eye examination. One drop of oxybuprocaine 0.4% was applied to each eye immediately prior to examination. BIO was performed using a 28 dioptre lens. A lid speculum and scleral indenter were used. WFDRI images were recorded with the RetCam II Digital Retinal Camera (Clarity Medical Systems) with the neonatal nose cone. A lid speculum was used and polyacrylic acid gel (viscotears) was applied to the anaesthetised cornea before capturing the images. The study coordinator timed how long each eye examination took from the insertion of the eyelid speculum to its removal at the end of the examination. All examinations were performed with cardiac and oxygen saturation monitoring. The examinations were interrupted if an infant's heart rate or oxygen saturation decreased to an unacceptably low level and recommenced once these had stabilised.

5.3.4 Clinical Findings

Both examiners graded retinopathy according to The International Classification of ROP [The International Classification of ROP revisited, 2005]. The stage of ROP, zone of vascularisation, number of clock hours of ROP and presence or absence of 'plus' disease was documented. 'Plus' disease represented significant dilatation and tortuosity of posterior pole blood vessels meeting or exceeding that of a standard photograph [The International Classification of ROP revisited, 2005]. 'Threshold' ROP, as defined in the CRYO-ROP trial referred to stage 3 ROP in zone 1 or 2 of 5 or more contiguous or 8 or more cumulative clock hours in the presence of 'plus' disease [Cryotherapy for ROP cooperative group, 1988]. All eyes with 'threshold' ROP were treated with diode laser therapy from June 2004-December 2004. From January 2005, new treatment criteria were used following the publication of the ETROP study [Early Treatment For Retinopathy Of Prematurity Cooperative Group, 2003]. Eyes with type 1 ROP were treated and both eyes were treated.

5.3.5 Examination documentation and management plans

Each examiner documented their findings in separate books and remained masked to the other examiner's findings and to their own findings from previous weeks. Based on their findings, each examiner documented a management plan, which was to

discharge the infant, treat the infant or examine the infant again in either 1 or 2 weeks time. The study coordinator read each examiners management plans. If there was any difference in management plan then the infant was seen again the following week. This continued until both management plans were the same. If a decision to treat was reached using BIO but not using WFDRI then the infant was seen the following week. If the infant still warranted treatment the following week as judged by BIO then the infant was treated even if a discrepancy remained with the WFDRI management plan. The study coordinator was present at all examinations to ensure that examiners remained masked to each other's findings.

5.3.6 Standardisation of examiners

The two specialised paediatric ophthalmologists have worked together using BIO to perform all ROP eye screening examinations in the Lothian region since 1990. In order to measure interobserver agreement, the examiners independently analysed RetCam images from 81 clinical ROP screening examinations from the study. The stage of ROP seen, the presence or absence of plus disease and the location of the disease were documented.

5.3.7 Data interpretation and statistical method

For each eye examined by BIO and WFDRI, the highest stage of ROP, the presence or absence of plus disease and the management plan were recorded and used for analysis. The WFDRI findings were compared to those of the current 'gold standard' of BIO and the sensitivity and specificity, together with 95% confidence intervals, were calculated. The WFDRI management plan reached for each infant after both eyes were examined was compared with the plan from the 'gold standard' BIO by calculating the kappa value as a marker of technique agreement.

5.4 Results

5.4.1 Patients and study examinations

A total of 81 babies were recruited from May 2004 to June 2007. The median gestational age at birth of study infants was 29 weeks (interquartile range 27 to 31) and median birth weight was 1225 grams (interquartile range 878 to 1492). A total of

123 eye examinations on both eyes were carried out (246 eyes examined in total). On one occasion it was not possible to obtain adequate quality images from one eye using WFDRI. Findings from 245 eyes were therefore analysed. 54 infants contributed one examination, 20 infants contributed two examinations, 3 infants contributed three examinations, 2 infants contributed four examinations, 1 infant contributed five examinations and 1 infant contributed seven examinations on both eyes. Not every examination of every infant was included as study examinations could not be performed when one examiner was unavailable. In this cohort, BIO detected 24 infants who developed ROP (24/81, 30%) and 5 infants (5/81, 6%) that required treatment. On 63/123 examinations BWF used WFDRI and EW used BIO.

5.4.2 Standardisation of examiners

The two examiners demonstrated 95% agreement (95% CI 91 to 98) on the presence or absence of plus disease, 94% agreement on stage of ROP (95% CI 91 to 98) and 97% agreement (95% CI 95 to 99) on the zone of ROP following the independent, masked scoring of 81 RetCam images. In addition, the study data were analysed for systematic bias between the two examiners in each diagnostic category, for BIO and for WFDRI. There were no significant systematic differences in any diagnostic category, using the Fisher exact test (table 5.1).

Table 5.1: Diagnostic comparison of the two examiners

	Examiner 1 BIO	**Examiner 2 BIO**	**Examiner 1 WFDRI**	**Examiner 2 WFDRI**
Plus disease (number of eyes)	5	5	7	4
Stage 3 ROP (number of eyes)	7	7	6	7
Any ROP (number of eyes)	26	19	25	21
Treat (number of infants)	3	3	4	3
Discharge (number of infants)	35	34	31	31

5.4.3 Detection of disease

The detection of any stage of ROP, stage 3 disease and plus disease is shown in tables 5.2-5.4.

Table 5.2: Detection of any stage of ROP

	BIO ROP present	BIO no ROP
WFDRI ROP present	27	19
WFDRI no ROP	18	181

Sensitivity=60% (44-74%). Specificity=91% (86-94%)

Table 5.3: Detection of stage 3 disease

	BIO stage 3 present	BIO no stage 3 disease
WFDRI stage 3 present	8	5
WFDRI no stage 3 disease	6	226

Sensitivity=57% (29-82%). Specificity=98% (95-99%)

Table 5.4: Detection of plus disease

	BIO plus present	BIO no plus
WFDRI plus present	8	5
WFDRI no plus	2	230

Sensitivity=80% (44-97%). Specificity=98% (95-99%)

5.4.4 Proportional agreement

The proportional agreement of the two examination methods is shown in table 5.5.

Table 5.5: Proportional agreement of WFDRI and BIO

Outcome	Proportional agreement
Detection of any stage of ROP	0.85
Detection of stage 3 disease	0.96
Detection of 'plus' disease	0.97

The proportional agreement is represented by the number of times that BIO and WFDRI concur divided by the total number of examinations.

5.4.5 Patient management decisions

Following the eye examination, each examiner decided a management plan for the infant choosing one out of three possible outcomes- treat the infant, discharge the infant or review the infant in 1 or 2 weeks time. The kappa value for agreement on management decisions between the WFDRI and BIO was 0.85 which is very good agreement. Details of decisions to treat and discharge are given in tables 5.6 and 5.7.

Table 5.6: Decision to treat an infant

	BIO treated	**BIO not treated**
WFDRI treat	5	2
WFDRI not treat	1	115

Table 5.7: Decision to discharge an infant

	BIO discharged	**BIO not discharged**
WFDRI discharge	56	5
WFDRI not discharge	14	48

On two occasions the WFDRI management decision was to treat the infant while the BIO decision was not to treat. One infant was re-examined the following week and both WFDRI and BIO agreed that treatment was required. The second infant was re-examined the following week using BIO only (one examiner was absent) and was not treated.

On one occasion the BIO decision was to treat the infant while the WFDRI decision was not to treat. The documented clinical findings for this individual case showed that the infant was borderline for treatment. When this infant was re-examined one week later both BIO and WFDRI agreed that treatment was required.

On five occasions the WFDRI decision was to discharge the baby and the BIO was to perform a further examination. On re-examination the following week there was agreement between WFDRI and BIO in two cases. The remaining three infants had left the study due to transfer back to local hospitals and detailed retinal examination

findings were unknown. However it was known that no infant subsequently required treatment. On fourteen occasions the BIO decision was to discharge the infant and WFDRI disagreed. There was agreement on management plan within one week in seven cases and two weeks in one case. We do not have information on five infants who left the study due to transfer back to local hospitals. It was known that none of these infants subsequently required treatment.

5.4.6 Time taken to complete an examination

The median time taken for WFDRI examination of both eyes was 110 seconds (interquartile range 80 to 133). The median time taken for BIO of both eyes was 90 seconds (interquartile range 65 to 120). Examinations using BIO were significantly quicker (Mann Whitney p=0.005).

5.5 Discussion

We compared the ability of WFDRI to detect ROP and guide patient management decisions compared to the current gold standard of BIO. We found the sensitivity of WFDRI in detecting any ROP, stage 3 ROP and plus disease to be 60%, 57% and 80% respectively with a specificity of 91%, 98% and 98% respectively. There was excellent proportional agreement between the two screening modalities for detecting stage 3 ROP and plus disease (0.96 and 0.97) and very good agreement on management decisions (kappa value 0.85).

There have been a number of other studies comparing the performance of WFDRI and BIO for ROP screening. These studies have used similar methodology whereby the same or different examiners perform WFDRI and BIO imaging on an infant on the same or different screening occasions. The stored WFDRI images are then anonymised and interpreted by either the study examiner or another specialist. One of the key strengths of our study lies in the methodology as we present, to our knowledge, the first masked, double-observer prospective randomised comparison of WFDRI and BIO for ROP examinations.

Previous studies have, like us, reported lower sensitivities and higher specificities for disease detection. Roth *et al* found 82% sensitivity and 94% specificity of WFDRI

compared with BIO for detecting any stage of ROP [Roth *et al*, 2001]. Shah *et al* found 86% sensitivity and 92% specificity of WFDRI compared with BIO in detecting any stage of ROP and no case of threshold ROP was missed with WFDRI [Shah *et al*, 2006]. Yen *et al* used WFDRI to attempt to predict which eyes would develop threshold disease by evaluating WFDRI images at two distinct gestational time points [Yen *et al*, 2002]. Low sensitivities and high specificities were recorded for detecting various stages of ROP. The authors of these three studies thought their lower sensitivities were due in part to the technical difficulties encountered using a standard child lens attachment for the screening examinations. A specialised smaller neonatal nose cone has now been developed which we used in our study.

Despite using the neonatal nose cone, our ophthalmologists still reported technical difficulties in imaging the peripheral retina using WFDRI. Both ophthalmologists agreed that visualisation of the peripheral retina was easier and more complete using BIO rather than WFDRI. The stages of ROP are predominantly present in zones 2 and 3 which are more peripheral than posterior zone 1. This may explain our low sensitivities of WFDRI for detection of any stage of ROP and stage 3 ROP (60%, 57%) as this disease is likely to be located peripherally and therefore technically more difficult to capture using WFDRI. However, other studies using the neonatal nose cone have reported higher sensitivities for WFDRI detection of any stage of ROP.

The results from the ETROP study have led to changes in clinical ophthalmology practice worldwide [Early Treatment For Retinopathy Of Prematurity Cooperative Group, 2003]. This study identified plus disease as the new driver for ROP treatment. All eyes in which 'plus' disease is present (type 1 disease) should be treated for optimal visual outcomes. Therefore, any potential screening modality must be good at detecting 'plus' disease. 'Plus' disease is located in the posterior retina and WFDRI is particularly effective at imaging this region. We found that WFDRI was better at detecting plus disease (sensitivity 80%) than detecting stages of ROP. Other studies have reported much higher sensitivities for detecting 'plus' disease using WFDRI. Ells *et al* and Wu *et al* reported a sensitivity of 100% using WFDRI to

detect 'referral warranted' ROP (any zone 1 disease, 'plus' disease or stage 3 disease) [Ells *et al*, 2003; Wu *et al*, 2006]. Lorenz *et al* also reported a sensitivity of 100% for detecting suspected treatment ROP (type 1 ROP according to ETROP) using WFDRI in a large cohort of 913 infants [Lorenz *et al*, 2009]. The Stanford University Network for Diagnosis of ROP (SUNDROP) study also found a sensitivity of 100% for WFDRI detection of ROP that required treatment [Murakami *et al*, 2009].

Our calculated specificities for using WFDRI to detect ROP were very high and our calculated sensitivities were lower. We studied a consecutive series of infants and stage 3 ROP and plus disease were uncommon in our study population. This meant that a discrepancy in detection of disease between WFDRI and BIO in just one case led to a marked reduction in sensitivity with minimal change in specificity.

Although the sensitivity and specificity of WFDRI in detecting ROP is important, the management decisions made in light of viewing the images are critical. In our study, all cases judged to require treatment following BIO examination were identified with WFDRI. One infant, with borderline findings, was thought to require treatment following BIO but not following WFDRI. However, one week later a decision to treat was made by both observers. In our study the sensitivity of WFDRI in making treatment decisions was therefore 100%, allowing for a repeat examination after one week. Interestingly, following WFDRI a management decision on two occasions was to treat the infant a week before BIO examination resulted in a management decision to treat. Ells *et al* found that severe ROP was diagnosed by WFDRI at least 1 week before BIO in 43% of eye examinations [Ells *et al*, 2003].

In our study there were 14 occasions when the management plan to discharge an infant was reached using BIO but not reached using WFDRI. This was likely due to better visualisation of the peripheral retina and hence better visualisation of normal vascularisation into zone 3 using BIO than WFDRI. This suggests that BIO is superior to WFDRI in making decisions to discharge infants from ROP screening.

The quality of the images obtained and technical usability of WFDRI has been reported to be variable. Wu *et al* failed to interpret 21% of screening examinations due to poor image quality, necessitating referral for BIO [Wu *et al*, 2006]. Ells *et al* failed to perform WFDRI on 4% of infants due to technical difficulties [Ells *et al*, 2003]. Motion artefact is also a well documented problem when using WFDRI. In the multi-centre Photo-ROP study, 8% of image sets were uninterpretable [The photographic screening for ROP study, 2008]. We encountered minimal problems with our ophthalmologists failing to obtain only one adequate quality WFDRI eye examination (1/246 eyes: 0.4%).

We found that WFDRI took longer to perform than BIO (median time WFDRI 110s *vs.* BIO 90s). This may change as examiners become more experienced and familiar with WFDRI. Some centres have developed a protocol for WFDRI for ROP and this again may help reduce WFDRI examination time.

5.6 Summary

We have carried out a masked, double-observer prospective randomised comparison to determine the sensitivity and specificity of WFDRI in detecting and making ROP management decisions, compared to the current gold standard of BIO. WFDRI showed relatively poor sensitivity for detecting mild forms of ROP located in the peripheral retina but was more effective at detecting 'plus' disease in the posterior retina. WFDRI correctly identified all infants that required laser therapy for ROP. BIO was superior to WFDRI in making decisions to discharge infants from the screening programme. As a result of our study, our ROP screening practice has changed in our neonatal unit. WFDRI is currently used for ROP screening examinations and an anticipated final 'discharge' examination of an infant is carried out using BIO.

CHAPTER 6

PAIN IN NEONATES DURING SCREENING FOR RETINOPATHY OF PREMATURITY USING BINOCULAR INDIRECT OPHTHALMOSCOPY AND WIDE-FIELD DIGITAL RETINAL IMAGING

Dhaliwal CA, Wright E, McIntosh N, Dhaliwal K, Fleck BW.
Pain in neonates during screening for retinopathy of prematurity using binocular indirect ophthalmoscopy and wide-field digital retinal imaging: a randomised comparison.
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6.1 Introduction

Extremely preterm infants need regular screening for the early detection of retinopathy of prematurity (ROP) and the successful prevention of its blinding end stage [Royal College of Paediatrics and Child Health, 2008]. The mainstay of screening is binocular indirect ophthalmoscopy (BIO) with eyelid speculum and scleral indentation which improves examination of the peripheral retina [Dhillon *et al*, 1993]. It is recognised as being an uncomfortable and distressing procedure and topical anaesthesia should be applied immediately prior to screening [Royal College of Paediatrics and Child Health, 2008]. Comfort care (using pacifiers, sucrose or nesting) is used more variably, although evidence suggests that this can ameliorate the pain [Kleberg *et al*, 2008; Gal *et al*, 2005; Mitchell *et al*, 2004]. Systemic analgesia is not normally given.

The use of the digital retinal camera for wide-field digital retinal imaging (WFDRI) has been suggested as a replacement for BIO. WFDRI can be carried out by less specialist staff with the help of experts for interpretation using telemedicine. It has been suggested that WFDRI may be less painful than BIO [Mukherjee *et al*, 2006]. Chapter 5 reports on our randomised comparison of the diagnostic accuracy of WFDRI with BIO. We designed, as part of our investigation, a study to compare the pain effects of both BIO and WFDRI screening processes and this chapter describes the findings.

6.2 Aim

To compare the pain experienced by premature infants undergoing WFDRI and BIO for ROP screening.

6.3 Methods

This was a prospective, randomized, comparative study. Ethical approval was granted by the Lothian Research Ethics Committee and all parents gave written consent.

6.3.1 Subjects and study design

All infants recruited required routine ROP screening at Edinburgh Royal Infirmary Neonatal Intensive Care Unit between June 2004 and May 2007. Infants were excluded if they were requiring mechanical ventilation or analgesic medication or if they had moderate/severe neurological impairment (grade3/4 intraventricular haemorrhage or periventricular leukomalacia) as it was felt that these infants would respond differently to painful stimuli. Infants were examined by two experienced paediatric ophthalmologists (BF and EW). Only the first screening examination for each baby was included in this study. Infants had both eyes examined by both WFDRI and BIO. The study coordinator (CD) randomised both examiners to screening using either WFDRI or BIO and also randomised the order in which the examinations were to be carried out. The right eye was always examined first.

6.3.2 Eye examinations

Infants had their pupils dilated with topical phenylephrine 2.5% and tropicamide 0.5% applied 60 minutes and 30 minutes prior to eye examination. Infants were then handled minimally and were not exposed to painful procedures unless medically indicated. One drop of oxybuprocaine 0.4% was applied to each eye immediately prior to each eye examination. Infants were examined on a cot blanket. They were unswaddled and non-nested. The study coordinator held the infants arms gently across their chests and steadied their heads during both examinations. Pacifiers were not used and oral sucrose was not given. A lid speculum (Barraquer Oosterhuis child speculum) and scleral indenter (Schokett style paediatric scleral depressor) were routinely used during BIO. WFDRI images were taken using the digital retinal camera (RetCam II, Clarity Medical Systems) with the neonatal nose-cone. The same type of lid speculum was used but scleral indentation was not routinely performed. After the first examination by the first examiner, the infant was handled minimally and allowed to recover for at least 30 minutes, to allow physiological variables to return to baseline prior to undergoing the second eye examination. All infants had constant cardiac and oxygen saturation monitoring.

6.3.3 Pain Monitoring and data interpretation

The Premature Infant Pain Profile (PIPP) scoring system (table 6.1) was used [Stevens *et al*, 1996]. Infants were observed for 15 seconds immediately prior to each eye examination (before insertion of oxybuprocaine eyedrops) and their baseline heart rate, oxygen saturation and behavioural state was noted. During the first minute of the right eye examination the maximum and minimum heart rate and minimum oxygen saturation of the infants were documented. The facial features of every infant were video-recorded and videotapes were later reviewed by an independent observer (KD) who scored the facial features during the first minute of BIO and WFDRI according to the PIPP. KD could not be blinded to the type of examination. The PIPP scores, heart rates and oxygen saturations for WFDRI and BIO were compared using paired t tests generated from the GraphPad InStat program. Fisher's Exact Test was used to compare the occurrence of marked bradycardias and desaturations. A p-value <0.05 was taken to indicate statistical significance.

Table 6.1:PIPP scoring system [Stevens *et al*, 1996].

Indicator	Finding	Points
Gestational age	>/=36 weeks	0
	32 weeks-35 weeks 6 days	1
	28 weeks-31 weeks 6 days	2
	<28 weeks	3
Behavioural state	active/awake eyes open facial movements	0
	quiet/awake eyes open no facial movements	1
	active/sleep eyes closed facial movements	2
	quiet/sleep eyes closed no facial movements	3
Heart rate maximum	0-4 beats per minute increase	0
	5-14 beats per minute increase	1
	15-24 beats per minute increase	2
	>/= 25 beats per minute increase	3
O2 saturation minimum	0-2.4% decrease	0
	2.5-4.9% decrease	1
	5.0-7.4% decrease	2
	7.5% decrease or more	3
Brow bulge	none (</=9% time)	0
	minimum (10-39% time)	1
	moderate (40-69% time)	2
	maximum (>/=70% time)	3
Eye squeeze	none (</=9% time)	0
	minimum (10-39% time)	1
	moderate (40-69% time)	2
	maximum (>/=70% time)	3
Nasolabial furrow	none (</=9% time)	0
	minimum (10-39% time)	1
	moderate (40-69% time)	2
	maximum (>/=70% time)	3

6.4 Results

A total of 81 infants were recruited to our WFDRI/BIO diagnostic comparative trial. Inadequate quality video-recordings were obtained from four infants and one infant was excluded as he had periventricular leukomalacia. Therefore, a total of 76 infants were included in this study of whom 39 received BIO first and 37 WFDRI first. The baseline characteristics of the study population are shown in table 6.2. There were 40 male infants and 36 females. A total of 50 infants were breathing room air, 13 infants had nasal prong oxygen and 13 infants were having nasal continuous positive airway pressure with oxygen.

Table 6.2: Baseline characteristics of the study population

	Mean (range)
Gestational age (weeks)	28.6 (24-35)
Corrected gestational age at ROP examination (weeks)	34.1 (30-40)
Birth weight (grams)	1208 (610-1970)
Number of days intubated and ventilated	4.6 (0-45)
Number of days of morphine	0.2 (0-5)

Table 6.3 shows details of infants' heart rates and oxygen saturation levels before and during both eye examinations. There were no differences in baseline levels between the two groups. The mean PIPP score for WFDRI was 15.0 (SD 2.1) and mean PIPP score for BIO was 15.2 (SD 2.4). There was no statistically significant difference in PIPP scores (paired t test p=0.47).

Table 6.3: Heart rate and oxygen saturation details

	BIO mean (SD)	**WFDRI mean (SD)**
Baseline heart rate (bpm)	148 (13)	147 (14)
Minimum heart rate during examination (bpm)	134 (25)	133 (28)
Maximum heart rate during examination (bpm)	168 (18)*	172 (17)*
Baseline oxygen saturation (%)	96 (4)	96 (4)
Minimum oxygen saturation during examination (%)	87 (12)	88 (10)

* Statistically significant difference comparing BIO and WFDRI maximum heart rates (paired t test p=0.03)

We observed a significantly greater increase in heart rate during WFDRI than during BIO (p=0.03). During WFDRI, 8 infants had a marked bradycardia (HR<100bpm) and 11 infants had a marked desaturation (min O2 sat <80%). During BIO, 9 infants had a marked bradycardia and 15 infants had a marked desaturation. There was no statistically significant difference in occurrence of marked bradycardias (Fisher's Exact Test p=1.00) or marked desaturations (Fisher's Exact Test p=0.52) during the two examination techniques.

6.5 Discussion

This study, where infants received BIO and WFDRI, demonstrates that ROP eye screening is painful but the pain experienced is similar for both techniques.

The PIPP was chosen as it is a well validated method for measuring procedural pain in preterm infants and uses contextual indicators (gestational age and behavioural state), physiological indicators (heart rate and oxygen saturation) and behavioural indicators (brow bulge, eye squeeze and nasolabial furrow) in its calculation [Stevens *et al*, 1996]. The major drawback of using the PIPP score in this setting is that it only takes into account an increase in heart rate but bradycardia during eye screening can occur due to the oculocardiac reflex [Clarke *et al*, 1985]. We therefore additionally recorded the minimum heart rates during all examinations but there was no significant difference between the two screening methods.

Several studies have investigated the systemic effects of ROP screening using BIO by measuring physiological markers (heart rate, oxygen saturation and blood pressure) [Belda *et al*, 2004; Rush *et al*, 2004; Laws *et al*, 1996]. Laws *et al* report a median drop in oxygen saturation of 3% with a rise in heart rate of 7 beats per minute during BIO [Laws *et al*, 1996]. Rush *et al* also recorded a significant increase in heart rate and fall in oxygen saturation levels during the procedure [Rush *et al*, 2004]. Our results too show a reduction in oxygen saturation and increase in heart rate during both screening methods. In addition to studying physiological and behavioural parameters, Kleberg *et al* measured a biochemical parameter (salivary cortisol) during and after ROP eye screening using BIO [Kleberg *et al*, 2008]. Infants

were randomly assigned to receiving either Newborn Individualized Developmental Care and Assessment Program (NIDCAP) care or standard care during ROP screening examinations. They found no difference in physiological parameters or PIPP scores between the two care strategies during eye examination but lower salivary cortisol levels 60 minutes after examination in the infants receiving NIDCAP care, thus implying a faster recovery for these infants. It would be interesting to compare cortisol levels in infants undergoing BIO with infants undergoing WFDRI.

Mukherjee *et al* compared only cardiorespiratory parameters as a measure of distress during ROP screening using both BIO and WFDRI [Mukherjee *et al*, 2006]. In contrast to our study, they found a significantly higher increase in heart rate and respiratory rate in the group undergoing BIO than those undergoing WFDRI. This finding may be related to differences in our study populations and examination techniques and lack of randomisation in their study could have led to significant baseline variations and selection bias. Mehta *et al* carried out a pilot crossover study in a small cohort of twelve infants comparing WFDRI, BIO with speculum and BIO without speculum [Mehta *et al*, 2005]. They studied both physiological and behavioural parameters and although the cohort was too small to draw absolute conclusions, infants undergoing WFDRI and BIO with speculum had more pronounced changes than with BIO without speculum. Kirchner *et al* compared pain scores and heart rates in a cohort of 92 infants who underwent either BIO with speculum and scleral indentation or BIO with no speculum and no scleral indentation. They also found that BIO without speculum and without scleral indentation was less stressful for the infants [Kirchner *et al*, 2009].

While carrying out this study we observed that infants immediately started crying with corresponding physiological changes as soon as the eyelid speculum was inserted and crying stopped on speculum removal. As both WFDRI and BIO require speculum use to obtain optimal retinal views we propose that the speculum, rather than the examination method may contribute most to the pain experienced. As discussed, other groups have also reported marked physiological responses

associated with speculum insertion [Mehta *et al*, 2005; Kirchner *et al*, 2009] and Slevin *et al* report marked neurobehavioral changes associated with speculum withdrawal [Slevin *et al*, 1997]. Perhaps more investigation should be directed to examination techniques that do not require the use of a speculum, or to improved speculum design and insertion technique. We are currently investigating the use of a small, relatively malleable Barraquer-style single-use neonatal speculum that can be slightly bent prior to use in order to set the blades closer together or wider apart in order to accommodate differing palpebral aperture sizes (Malosa Medical 7.00 mm closed blade, 0.8 mm wire; Malosa, Sowerby Bridge, UK).

Observations of infants undergoing eye screening by both WFDRI and BIO indicate that, despite use of topical anaesthesia and comfort care, these procedures remain painful and distressing for neonates [Sun *et al*, 2010]. Many extremely preterm infants need to be screened over a number of weeks. Exposure to repeated painful procedures has been shown to have long-term detrimental consequences [American Academy of Pediatrics, 2000]. These include neuroanatomical abnormalities, altered pain sensitivity, emotional, behavioural and learning disabilities [Oberlander *et al*, 2000; Ruda *et al*, 2000; Anand *et al*, 1999; Porter *et al*, 1999; Anand and Scalzo, 2000; Fitzgerald and Beggs, 2001; Maroney, 2003]. Effective analgesia can reduce altered pain sensitivity [Grunau *et al*, 2001; Peters *et al*, 2003]. Neonatal staff have an ethical obligation to minimize pain in neonates. There is an urgent need for studies to evaluate the use of different systemic analgesia regimes in order to optimise pain control during ROP screening.

6.6 Summary

This study demonstrates that ROP eye screening using both WFDRI and BIO with eyelid speculum and topical anaesthesia is painful for infants but the pain experienced is of a similar severity for both techniques. Further work is required to implement adequate analgesia regimes for ROP screening.

CHAPTER 7

GENERAL DISCUSSION

7.1 Summary of findings

7.1.1 Incidence of retinopathy of prematurity in Lothian, Scotland

Lothian population data showed a steady decline in the number of live births from 1990-2004. The proportion of babies born with birth weight (BW) <1500g and/or gestational age (GA) <32 weeks remained constant (p=0.271 using chi-square test), though the proportion of these babies surviving to 42 weeks corrected gestation increased from 1990-2004 (p<0.001 using chi-square test for trend). There was a statistically significant linear trend towards a reduction in the number of babies undergoing treatment for retinopathy of prematurity (ROP) throughout the study period (p<0.01 using chi-square test for trend). A reduction in the incidence of any degree of ROP and severe (stage 3 or greater) ROP was also observed although this did not reach statistical significance.

In summary, there was a significant increase in survival of infants with BW<1500g and/or GA<32weeks together with a significant reduction in the number of infants treated for ROP in the Lothian region of South East Scotland from 1990-2004.

7.1.2 ROP in small-for-gestational age infants

A total of 1413 babies with birth weights <1500g and/or gestational age <32 weeks underwent eye screening in the Lothian hospitals from 1990-2004. 329/1413 (23%) of the study population was small-for-gestational age (SGA). SGA infants born at gestational ages 26-31 weeks were more likely to develop any stage of ROP (p<0.01) than their appropriate-for-gestational age (AGA) peers. SGA infants were also more likely to develop severe ROP (GA 26-27wks p<0.01, GA 28-29wks p=0.01, GA 30-31wks p=0.01).

In summary, SGA infants who underwent eye screening in the Lothian region of South East Scotland from 1990-2004 were significantly more likely to develop ROP and more severe disease than AGA infants.

7.1.3 The influence of growth, insulin-like growth factor-1 and oxygen on retinal vascular development

This was a laboratory based animal study. Rat pups were growth restricted in the prenatal and postnatal periods. This was carried out by feeding the dams a low protein (9%) diet. Litters of growth restricted pups and litters of normal sized pups were exposed to either room air or to a variable oxygen profile (minute-minute fluctuations around a mean inspired oxygen concentration of 24%), known to induce retinal changes similar to clinical ROP.

In summary, retinal blood vessel development in growth restricted pups was slower than in normal sized pups. Retinal blood vessel development in pups exposed to variable oxygen was slower than in pups exposed to room air. Growth restricted pups had lower serum insulin-like growth factor-1 (IGF-1) levels than normal sized pups and pups that were slower to gain weight had lower serum IGF-1 concentrations.

We suggest that serum IGF-1 concentration may have a direct effect on retinal vascularisation in our rat model of ROP, with a low serum IGF-1 being associated with slower retinal vascular development. Our work demonstrates that prenatal and postnatal nutrition is important to promote optimal retinal blood vessel development and supports our clinical finding that small-for-gestational age infants have more severe ROP than appropriately sized peers.

7.1.4 Wide-field digital retinal imaging versus binocular indirect ophthalmoscopy for ROP screening

This was a prospective, randomised, comparative study. A total of 81 infants were enrolled and results from 245 eyes were analysed. The sensitivity of wide-field digital retinal imaging (WFDRI) in detecting any ROP, stage 3 ROP and plus disease was 60%, 57% and 80% respectively with a specificity of 91%, 98% and 98% respectively. There was excellent proportional agreement between WFDRI and binocular indirect ophthalmoscopy (BIO) for detecting stage 3 ROP and plus disease (0.96, 0.97). There was very good agreement between the two screening methods on management decisions (kappa value 0.85).

In summary, WFDRI was good at detecting plus disease located at the posterior pole. BIO was better than WFDRI at detecting stages of ROP which are predominantly found in the more peripheral retina. WFDRI identified all infants that required treatment for ROP. WFDRI is a useful examination technique for ROP screening.

7.1.5 Pain in neonates undergoing ROP screening

A total of 76 infants were recruited in this prospective, randomised, comparative study. The mean Premature Infant Pain Profile (PIPP) score for WFDRI was 15.0 (SD 2.1) and for BIO was 15.2 (SD 2.4). There was no statistically significant difference in occurrence of marked bradycardias or marked desaturations (Fisher's exact test p=1.00, p=0.52 respectively) during the two screening techniques. We observed that infants started crying with corresponding physiological changes as soon as the eyelid speculum was inserted and crying stopped on speculum removal.

In summary, despite application of topical anaesthesia, WFDRI and BIO with eyelid speculum are painful procedures. The pain experienced is of similar severity for both techniques. We propose that the eyelid speculum, rather than the examination technique, contributed most to the pain experienced.

7.2 Limitations of data

7.2.1 Incidence of ROP in Lothian, Scotland

The main limitation of this work was the discrepancy found between the Information Services Division (ISD), Scotland epidemiological data and our own hospital data (186 babies). We were unable to cross-check our records with ISD due to patient confidentiality and data protection legislation. It is likely that these 186 babies were either transferred back to their local hospitals or were discharged home and failed to attend outpatient eye screening. Ethnicity is a well documented risk factor for ROP with Asian and Afro-Carribean infants being at greater risk of severe ROP [Aralikatti *et al*, 2009]. We did not formally collect ethnicity data and this information would

have been useful to allow readers of our paper to compare the baseline characteristics of our population with their own.

7.2.2 ROP in SGA infants

We studied the incidence of ROP in SGA infants which we defined as infants with a birth weight less than the 10^{th} percentile for gestational age. By definition, SGA refers only to size at birth and does not give us any information about fetal growth velocity. Within our SGA babies there will be infants with many different patterns of growth. Some will be constitutionally small with 'normal' patterns of growth while others will have been following a larger growth percentile until exposure to placental insufficiency in the third trimester resulting in growth failure. Our main limitation therefore refers to the terminology 'SGA' which is really a 'blanket term' referring to small babies as a result of many diverse underlying mechanisms. In order to address this issue and identify only the infants with growth failure, other researchers have used absent or reversed end diastolic flow in the umbilical artery as a marker of growth restricted babies. Another limitation of our study was we only looked at size at birth (SGA/AGA) and association with ROP and did not study rate of fetal growth or postnatal growth of SGA/AGA babies.

7.2.3 The influence of growth, IGF-1 and oxygen on retinal vascular development

Our animal model is a useful tool to study the pathogenesis of ROP but caution is required when relating findings to the human premature infant. Our rat model, although unique and very physiological, has some limitations. Mainly, the model does not cause frank neovascularisation. Most other animal models developed cause retinal neovascularisation and retinal grading systems have been developed to score the degree of neovascularisation together with specific processing and staining techniques used to quantify this [Zhang *et al*, 2000; Penn and Thum, 1989]. We were unable to use these and therefore unable to directly compare our findings with others. Unfortunately we were not able to relate individual rat pup retinas to the corresponding pup serum IGF-1 and pup weight. This was due to technical difficulties in retinal dissection, staining and in some cases low volume of serum obtained.

7.2.4 Wide-field digital retinal imaging versus binocular indirect ophthalmoscopy for ROP screening

The strength of our study lay in the study design. By using two expert ophthalmologists using the two screening techniques we reduced intraobserver bias which was a major flaw in previously reported studies [Roth *et al*, 2001; Shah *et al*, 2006; Chiang *et al*, 2007; Ells *et al*, 2003]. In order to check interobserver agreement between our two ophthalmologists they independently analysed WFDRI images and we reported good agreement. We did not however check interobserver agreement between our ophthalmologists using BIO. This is a limitation of our study. In order to do this we would have had to allow both ophthalmologists to perform BIO on the same infant on the same screening occasion and compare their findings. We would have required separate ethical approval for this and we felt that this was not necessary as both ophthalmologists had worked together using BIO for ROP screening for over ten years and had shown good interobserver agreement on WFDRI images. We felt that subjecting infants to an additional stressful examination could not be justified ethically.

A key strength of our study was that we recruited consecutive infants. However we included all infants eligible for eye screening and by doing so had a high proportion of more mature infants with mild disease and fewer lower gestational age infants with more severe disease. BIO detected stage 3 ROP in 14/245 (6%) eyes and plus disease in 10/245 (4%) eyes. As a result, any discrepancies between WFDRI and BIO in disease detection in just one case had a large influence on sensitivity and negligible influence on specificity. The prevalence of ROP increases with decreasing gestational age. Perhaps future comparative studies should recruit consecutive infants with lower gestational ages, for example those <28 weeks gestation.

7.2.5 Pain in neonates undergoing ROP screening

We chose to use the PIPP score as it is a well validated method of scoring procedural pain in premature infants [Stevens *et al*, 1996]. The one limitation of this score for ROP screening is due to the fact that neonates often experience bradycardias during screening caused by the oculocardiac reflex [Clarke *et al*, 1985]. The PIPP score

does not take this into account. We tried to overcome this limitation by documenting the lowest heart rates recorded on all infants. I think the study could have been improved by performing sequential PIPP scores, perhaps every minute during eye examination and during the recovery. This would have given us more information on the time required to re-attain baseline PIPP scores as perhaps the pain of one method may be more prolonged than the other. In order to prove our theory that the eyelid speculum is the cause of most pain, a PIPP score immediately prior to speculum insertion and immediately post speculum removal would have been beneficial. Ideally this study should have had one ophthalmologist performing all the eye examinations, in order to eliminate inter-examiner variability in examination handling and technique, but this was not possible as this data collection was integrated into our WFDRI versus BIO comparative study.

7.3 Future directions

7.3.1 Epidemiology of Retinopathy of Prematurity

The Early Treatment for ROP (ETROP) study has been one of the key publications on ROP in the last decade and has led to widespread change in clinical practice [Early Treatment for ROP Cooperative Group, 2003]. Infants with ROP less severe than threshold are now being treated. This especially applies to infants with stage 2 disease in zone 2 with plus disease. In addition, the severity of disease in zone 1 that requires treatment has been better defined. The presence or absence of 'plus' disease is now the key factor in determining if an infant requires treatment. There is a need to report on the incidence and severity of ROP since the introduction of these new treatment indications and changes in practice, as the natural history of the disease is now interrupted by treatment at an earlier stage in some infants.

The incidence of ROP is closely associated to neonatal oxygen exposure [Vanderveen *et al*, 2006; Wallace *et al*, 2007; Wright *et al*, 2006; Carlo *et al*, 2010]. Despite decades of research on this association, optimal neonatal oxygen saturation targets have yet to be established. A recent large randomized trial comparing target oxygen saturations of 85-89% or 91-95% in preterm infants identified an increase in

mortality in the lower-oxygen-saturation group [Carlo *et al*, 2010]. If confirmed, we have hit a barrier to further reduction of ROP by oxygen management. The BOOST-II UK is a double blind randomised controlled trial comparing the effects of targeting oxygen saturation levels of 85-89% versus 91-99% in infants <28 weeks gestation and will help to clarify the situation [National Perinatal Epidemiology Unit, 2009]. There is an urgent need to define appropriate oxygen saturation ranges in order to minimize ROP but without increasing adverse outcomes. There is now even more incentive to find other ways of reducing the incidence of ROP, for example, by improving nutrition.

Population based longitudinal studies provide valuable information on changes in disease and clinical practice over time. This enables objective auditing of care leading to improvements in clinical effectiveness. It would be advantageous to develop both a Scottish and British national Neonatal Intensive Care Units database as this would provide important information for planning future research, allocating resources and designing public health policies relating to ROP and all other neonatal diseases.

Screening programmes for ROP are well established in industrialised countries and provide vital information on the population of babies requiring treatment and the changes in this population over time. This information is used to refine screening and treatment criteria. There is now an urgent need to collect data on the population of babies developing ROP in 'middle-income' countries. This data will enable the implementation of relevant screening programmes in these countries where ROP blindness is increasingly prevalent.

7.3.2 Growth, nutrition and Retinopathy of Prematurity

Survival rates of premature infants have improved and more infants are at risk of developing ROP. There is therefore a need to develop effective strategies to reduce the morbidity following preterm birth.

Many ROP therapies aim to inhibit pathological neovascularisation, the late stage of the disease, rather than trying to improve normal vascular development. There is a window of opportunity in the early weeks following birth before ROP develops in which to a preventative therapy could be administered. Many infants treated by laser photocoagulation do not develop optimal visual acuity. Development of a pharmacological therapy that would improve normal retinal vascular development and thereby prevent neovascularisation and associated visual morbidity is highly desirable. The insulin-like growth factor system has potential to be manipulated as a preventative therapy.

IGF-1 and IGFBP-3 play critical roles in vascular development and administration of recombinant human IGF-1 or combined IGF-1/IGFBP-3 remains a potential preventative therapy. IGFBP-3 may be a novel agent to improve vascular development. As well as serving as a transporter protein for IGF-1, it has also been shown to be important in cell signalling affecting cell mobility and survival [Granata *et al*, 2004; Lofqvist *et al*, 2007]. Evidence is mounting in support of IGFBP-3 as an important regulator of both physiological and pathological angiogenesis and further research is required in order to identify the optimal preventative agent in the IGF system to target.

Early restoration of IGF-1 levels may be achieved through improving nutrition (in particular protein intake) or by exogenous administration [Smith *et al*, 1997]. Although nutrition with breast and formula milk will increase circulating IGF-1 levels, enteral nutrition in preterm infants may not be tolerated due to gut immaturity and concern about the risk of necrotising enterocolitis [Neu, 2007; Smith *et al*, 1997]. Exogenous administration may therefore be preferable. Animal studies are evaluating their use but further work is required to decipher optimal dosing, timing and route of administration regimens [Liechty *et al*, 1999].

Timing of any potential preventative therapy is crucial. Early administration of IGF-1 may improve normal retinal vascular development and thus prevent ROP but if administered too late, when the retina is hypoxic, it may potentiate

neovascularisation. Care must also be taken to minimize adverse effects on other organ systems [Mathews *et al*, 1988].

Poor weight gain during the early postnatal weeks is now being recognised as one of the strongest predictors for ROP [Lofqvist *et al*, 2006; Hellstrom *et al*, 2009]. Rate of postnatal weight gain together with serum IGF-1 levels have been used to predict which babies will develop ROP [Lofqvist *et al*, 2006; Hellstrom *et al*, 2009; Lofqvist *et al*, 2009]. The algorithm 'Weight IGF-1 Neonatal ROP' (WINROP) has been validated retrospectively, using just serial weight measurements, in Sweden and America with promising results [Hellstrom *et al*, 2009; Wu *et al*, 2010]. We are currently involved in validating the WINROP algorithm in Edinburgh using a ten year cohort of infants screened. This is exciting as it has potential to reduce the number of infants exposed to stressful eye examinations and suggests that improved nutrition in the early postnatal weeks, leading to improved weight gain, may reduce the risk of ROP from developing. We have found however, that very premature infants are often not weighed for several weeks due to the difficulties and risks involved in transferring a very sick ventilated infant from the incubator to the weighing scales. A better measure of linear growth is obtained using knemometry and this is an easier measurement to obtain in sick premature neonates [Gibson *et al*, 2003]. Further studies to look weekly knemometry readings and development of ROP are required.

Premature infants lack the long chain polyunsaturated fatty acids-omega-3 and omega-6 [Crawford *et al*, 2003]. Research suggests that lack of omega-3 may be associated with poor weight gain and ROP [Fierro *et al*, 2002; Kermorvant-Duchemin *et al*, 2005]. There is therefore potential to supplement these either in the diet or in intravenous fat emulsions but further research is required to check neonatal tolerance and safety.

There is a real need to define optimal growth rates in preterm infants, and in particular small-for-gestational-age preterm infants and this will require further research. Impaired fetal and postnatal growth in term infants have been related to a

higher risk of ischaemic heart disease, impaired glucose tolerance, type II diabetes mellitus, obesity and hypertension [Barker, 1990; Barker *et al*, 2002; Eriksson *et al*, 2007; Eriksson *et al*, 2006; Eriksson *et al*, 2003; Eriksson *et al*, 2001; Salonen *et al*, 2009; Osmond *et al*, 2007]. Evidence suggests that growth restricted infants with a fast catch-up growth are most at risk of developing these diseases [Lucas, 2005]. These observations have critical implications for preterm infants. Evidence suggests that a faster postnatal growth rate in preterm infants may reduce the incidence of ROP but there is no data yet available on the effect this may have on the incidence of vascular diseases in later life.

7.3.3 Screening for Retinopathy of Prematurity

WFDRI is an attractive screening method but it is not yet universally accepted [Kemper *et al*, 2008]. There are some technical difficulties regarding its use. Studies have reported greater accuracy when using WFDRI on more mature infants with higher corrected gestational age. This has been attributed to improved image capture and improved image quality because more mature infants have larger palpebral fissures, larger eyes and less corneal and vitreous haze [Chiang *et al*, 2007; Wu *et al*, 2006; Yen *et al*, 2002]. There is scope for the manufacturers to refine the camera lens and head in order to improve image capture and image quality in premature infants. This may lead to improvements in reported sensitivities for ROP detection which may result in more widespread use and acceptance of WFDRI in ROP screening.

WFDRI does have some distinct technological advantages over BIO. Firstly, WFDRI may be superior at detecting plus disease. Publications by the International Classification of ROP have been produced to provide an international standard and reference for detection of ROP [The Committee for the Classification of Retinopathy of Prematurity, 1984, 2005]. In particular, the articles contain images of 'plus' and 'pre-plus' disease to help examiners interpret the level of dilation and tortuousity sufficient for diagnosing plus disease. The ability of an ophthalmologist to correctly identify pre-plus and plus disease is crucial as it is now the primary indication for laser treatment in ROP [Early Treatment For Retinopathy Of Prematurity Cooperative Group, 2003]. Despite the attempts to standardise and clarify severity of

ROP, interobserver agreement remains very variable, even among experts in the field [Darlow *et al*, 2008; Chiang *et al*, 2007; Wallace *et al*, 2008]. With increasing use of WFDRI, image libraries can be developed for educational and research purposes with potential to improve the uniformity of ROP diagnosis. In order to reduce subjectivity and increase objectivity in diagnosis of plus disease, computer-based tools have been developed. These take quantitative measurements from blood vessels captured by WFDRI and have the potential to augment clinical assessment and improve inter-observer consistency [Johnson *et al*, 2007; Chiang *et al*, 2008; Aslam *et al*, 2009]. These are distinct advantages of WFDRI over BIO.

Secondly, WFDRI with store-and-forward telemedicine has potential to improve the accessibility and quality of care and may be particularly useful in 'middle-income' countries where the incidence of ROP is rising and where ROP screening is not yet routinely performed [Field, 1997; Gilbert, 2008]. One of the greatest barriers to implementing this screening method is initial cost of equipment. Two studies have compared the cost-effectiveness of telemedicine WFDRI versus BIO for ROP screening and both studies found several telemedicine strategies to be more cost effective than current BIO practice [Castillo-Riquelme *et al*, 2004; Jackson *et al*, 2008]. These results are encouraging and likely to change in the next decade with advances in information technology and increasing use of large-scale store-and-forward applications in healthcare driving down costs [Grigsby and Sanders, 1998; Krupinski *et al*, 2002].

In summary, WFDRI has many distinct technological advantages over BIO but has some technical limitations which need to be improved [Lorenz *et al*, 2009; Wu *et al*, 2006]. It remains unresolved what is 'adequate' accuracy for WFDRI to be accepted as a screening method and whether BIO is a true 'gold standard' against which WFDRI should been compared [Scott *et al*, 2008]. There is a need for more objective and accurate methods for assessing vascular abnormalities in order to treat infants at the optimal time.

7.3.4 Neonatal pain management during ROP screening

ROP screening examinations remain vital in order to identify infants with sight-threatening disease but are painful and distressing procedures for neonates. Neonatal exposure to repetitive painful procedures can have long-term negative effects [Anand *et al*, 1999; Anand and Scalzo, 2000]. Adequate analgesic regimes are not currently being performed. So far the mainstay of analgesia has been topical anaesthesia and comfort care. Although routinely given, there is little evidence in the literature to suggest that topical anaesthesia reduces pain [Marsh *et al*, 2005; Saunders *et al*, 1993]. The drops numb the cornea and conjunctiva but will have no effect on the discomfort caused from the traction on the orbicularis orbis muscle and lateral canthal skin by the eyelid speculum. Studies reporting on comfort care methods including pacifier use, sucrose and swaddling have variable findings [Gal *et al*, 2005; Mitchell *et al*, 2004; Boyle *et al*, 2006]. Certainly there is most evidence to suggest that sucrose and swaddling does ameliorate the pain experienced during eye screening [Gal *et al*, 2005; Mitchell *et al*, 2004; Slevin *et al*, 1997; Kleberg *et al*, 2008]. In our experience, the use of pacifiers during ROP screening is difficult as the infants tend to be screaming too much to suck the pacifier.

There is an urgent need to recognise that current analgesic regimes are inadequate and to identify suitable alternatives. One way is to try and change the examination technique. We are now using an eyelid speculum with smaller blades that do not stretch the eyelids as widely apart. We have not formally assessed the infants' pain responses but generally feel they seem less distressed than they were during our study. The brightness of the light is likely to be another cause of distress and perhaps a reduction in this may be beneficial for the infants. Another way is try different systemic analgesia. One small uncontrolled study used remifentanil (very short acting synthetic opioid) intravenously to effectively reduce the pain from laser photocoagulation [Sammartino *et al*, 2003]. This may be a possibility for routine eye screening although intravenous access would be required. An opioid intranasal spray would be an attractive option to avoid intravenous cannulation in the well infants.

These differing approaches could be used in conjunction with comfort care to reduce neonatal pain. Further research in this area is needed in order to enable the development of evidence-based clinical practice guidelines on the management of pain during ROP screening.

7.4 Conclusion

Retinopathy of prematurity is a leading cause of childhood blindness. Advances in obstetric and neonatal care within the developed world over the last two decades are likely responsible for the increased survival of premature infants and the overall decreasing incidence of ROP seen in Lothian. Current treatment for ROP with laser photocoagulation prevents blindness but is destructive and treated children still suffer visual morbidity in childhood. A preventative therapy is highly desirable. This research has demonstrated that body growth and IGF-1 are important in the pathogenesis of ROP. We have highlighted that SGA infants are particularly at risk. Nutritional therapies may help by improving normal retinal blood vessel development and thereby preventing local tissue hypoxia. Clinical trials of nutritional therapies are needed as these offer real potential to reduce the incidence of sight-threatening ROP. Our work has helped pave the way for future clinical trials. WFDRI is a useful examination technique for ROP eye screening but has some technical limitations which need to be improved. Further work is required to implement adequate analgesia regimes for ROP screening. These published trials will help form the basis for future clinical intervention studies. The WINROP algorithm could potentially lead to a reduction in the number of infants undergoing stressful eye examinations and I look forward to validating this in our Edinburgh cohort of infants.

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APPENDIX

1.2 Immunohistochemistry solutions

1.2.1 Phosphate buffered saline (PBS)

To make 1 litre:- weigh out 7g NaCl, 3.4g Na_2HPO_4, 0.8g KH_2PO_4 [Sigma-Aldrich, UK] and make up to 1 litre with distilled water. Check pH 7.3 using pH meter.

1.2.2 PX

1M buffered saline with 1% triton X.

To make 100mls PX:- take 10ml of 10% triton X stock solution [BDH, UK] and make up to 100mls with 1M buffered saline.

1.2.3 Blocking buffer

5% swine serum [Vector Laboratories Ltd, UK], 0.1% bovine serum albumin [Sigma-Aldrich UK], 0.3% triton X [BDH UK].

To make 100mls blocking buffer:- take 5mls swine serum, 0.1g bovine serum albumin, 0.3mls triton X and make up to 100mls with 1M buffered saline

1.2.4 Mowiol Mounting Medium

Add 2.4g Mowiol [Calbiochem, Merck, UK] to 6g of glycerol [Sigma-Aldrich, UK]. Add 12mls distilled water and leave at room temperature overnight. Add 12mls 0.2M Tris [Sigma-Aldrich, UK] and heat to 50^0C for 1-2 hours until Mowiol dissolved. Centrifuge at 2000rpm for 15 minutes. Add 0.72g of 1,4-DABCO [Sigma-Aldrich, UK]. Aliquot and store at -20^OC.

1.3 Rat diets

The diet compositions tabulated on the following page were kindly supplied by Dr Simon Langley-Evans, University of Nottingham. The diets were made to these compositions and supplied to us by Special Diets Services, Edinburgh. The crude protein in each batch was analysed.

	18% casein diet (g/kg of diet)	9% casein diet (g/kg diet)
Casein (dry acid free)	180	90
Corn oil	100	100
Cornstarch	425	485
Cellulose	50	50
Sucrose	213	243
Vitamins [1]	5	5
Minerals [1]	20	20
Choline chloride	2	2
D,L-Methionine	5	5

[1]AIN76 vitamin mix and AIN76 mineral mix from Purina Mills, USA

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Incidence of retinopathy of prematurity in Lothian, Scotland, from 1990 to 2004

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► Additional tables are published online only at http://adc.bmj.com/content/vol93/issue6

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ABSTRACT

Background: To report the trends in incidence of retinopathy of prematurity (ROP) within Lothian, a geographically defined region in southeast Scotland over a 15-year period from 1990 to 2004.

Methods: This was a prospective observational study of all infants born with gestational age <32 weeks and/or birth weight <1500 g who were born to mothers resident in Lothian between 1 January 1990 and 31 December 2004. Eligible infants underwent eye screening by two experienced paediatric ophthalmologists (BF and EW). Lothian population data were obtained from the Scottish Health Service. The trends in survival rates, incidence and treatment of ROP were analysed from 1990 to 1994, 1995 to 1999 and 2000 to 2004.

Results: Lothian population data showed a steady decline in the number of live births from 1990 to 2004. The proportion of babies born with birth weight <1500 g and/or gestational age <32 weeks remained constant ($p = 0.271$ using χ^2 test), although the proportion of these babies surviving to 42 weeks corrected gestation increased from 1990 to 2004 ($p<0.001$ using χ^2 test for trend). There was a statistically significant linear trend towards a reduction in the number of babies undergoing treatment for ROP throughout the study period ($p<0.01$ using χ^2 test for trend). A reduction in the incidence of any degree of ROP and severe (stage 3 or greater) ROP was also observed although this did not reach statistical significance.

Conclusions: There was a significant increase in survival of infants with birth weight <1500 g and/or gestational age <32 weeks together with a significant reduction in the number of infants treated for ROP in the Lothian region of southeast Scotland from 1990 to 2004.

What is already known on this topic

- Many centres are now reporting a reduction in the incidence of retinopathy of prematurity (ROP).
- However very few population-based studies have been published which accurately report the longer-term trends in ROP incidence.

What this study adds

- This 15-year population-based study shows a significant increase in survival of infants with birth weight <1500g and/or gestational age <32weeks together with a significant reduction in the number of infants undergoing treatment for ROP.
- There was also a trend towards a reduced number of infants with any stage of ROP and severe (stage 3 or greater) ROP.

Retinopathy of prematurity (ROP) is a disorder of retinal vascular development in premature infants. It is a major cause of childhood blindness worldwide.[1] The trends in incidence of ROP over time have been the subject of much debate. Since ROP was first described in 1942,[2] there have been two notable epidemics within the developed world.[3–6] The first was seen in the early 1950s and was associated with exposure to high oxygen concentrations for prolonged periods of time.[3] The second epidemic developed in the 1980s and was related to increased survival of very low birth weight (BW<1500 g) infants associated with advances in neonatal medicine.[4–7] Many centres have reported a decline in the incidence of ROP in the 1990s and early 2000s.[8–11] Since then, there has been an increasing body of evidence demonstrating that a modest reduction in target oxygen saturation levels is associated with a reduction in the incidence of severe ROP.[12–15] There has, however, been a marked lack of population-based studies, which will report the longer term trends in incidence of ROP most accurately.[16–21]

We report the trends in incidence of ROP within Lothian, a geographically defined region in southeast Scotland over a 15-year period from 1990 to 2004.

MATERIALS AND METHODS

Subjects

This was a prospective observational cohort study. The population of the study consisted of all infants eligible for eye screening who were born to mothers' resident within the Lothian region of southeast Scotland during the study period of 1 January 1990 – 31 December 2004. All infants born with gestational age <32 weeks and/or birth weight <1500 g who survived until eye screening commenced were eligible. Eligible Lothian babies born outside Lothian and transferred back into Lothian during eye screening were included.

The total population of Lothian increased steadily from approximately 745 000 in 1990 to 790 000 in 2004. There were approximately 9800 livebirths in 1990 which decreased over the years to 8300 in 2004. During the study period infants were born at one of three hospitals:

- The Simpson Memorial Maternity Pavilion/the New Royal Infirmary, Edinburgh: Level 3 South East Scotland Regional Neonatal Unit

with over 6000 live births per year. Approximately 520 infants admitted to neonatal unit each year. Hospital moved to the site of the New Royal Infirmary, Edinburgh in summer 2002.

- The Eastern General Hospital, Edinburgh: Level 2 neonatal unit with approximately 2200 live births and 250 babies admitted to the neonatal unit each year. This hospital closed in April 1998.
- St Johns Hospital, Livingston: Level 2 neonatal unit with approximately 2400 live births and 170 admissions to the neonatal unit each year.

Nursing staff in these hospitals referred all eligible babies to the ophthalmology team. Ophthalmologists recorded the date of birth, sex, gestational age, birth weight and maximum severity of ROP reached in any one eye for every baby examined. This information was prospectively stored on a database. Each hospital throughout the period of study used oxygen saturation monitor thresholds of 86–94%.

Lothian population epidemiological data

Epidemiological data was obtained from the Information Services Division (ISD) of the Scottish Health Service, which accessed the Scottish Morbidity Record (SMR02). Their figures differ from our Lothian hospital data and our results are based on both sources. Unfortunately, we could not cross-check our data with ISD due to patient confidentiality and data protection legislation, thus a small discrepancy remains.

Eye examination schedule

Screening and treatment (if required) was carried out by two dedicated paediatric ophthalmologists (BF and EW). Eligible infants were first examined at 4–6 weeks chronological age, or 34 weeks corrected age, whichever was earlier. Screening was continued fortnightly until full retinal vascularisation. Examinations were performed weekly if "prethreshold" disease was found (see below for definition).

Eye examination technique

Pupils were dilated with topical phenylephrine 2.5% and tropicamide 0.5% applied 60 min and 30 min prior to eye examination. Indirect ophthalmoscopy was performed using a binocular indirect ophthalmoscope and 28 Dioptre lens. A lid speculum and scleral indenter were routinely used. The entire retina was examined, including the periphery throughout 360°. Retinopathy was graded according to the International Classification of ROP.[22] The stage of ROP (1–5), zone of vascularisation, number of clock hours of ROP and presence or absence of "plus" disease was documented in both the eye logbook and patient medical notes. "Plus" disease represented significant dilatation and tortuosity of posterior pole blood vessels.[22] "Threshold" ROP referred to five or more contiguous or eight or more cumulative clock hours of stage 3 ROP in zones 1 or 2 in the presence of "plus" disease.[22] "Prethreshold" disease consisted of any stage 3 disease less extensive than threshold, or stage 2 disease in the presence of "plus".[22] All eyes examined with "threshold" ROP were treated with cryotherapy in 1990–1991 and with diode laser therapy from 1992 onwards. From January 2005 new treatment criteria were used following the publication of the Early Treatment For Retinopathy Of Prematurity (ETROP) study.[23] The study was therefore terminated at the end of 2004.

Statistical analysis

For analysis, the maximum severity of ROP in either eye for an individual infant was recorded. The 15-year study period was divided into three 5-year epochs: 1990–4, 1995–9 and 2000–4. Statistical analysis was performed using the GraphPad InStat programme (GraphPad Software, California, USA). Contingency tables were analysed using the χ^2 test (χ^2) and χ^2 test for trend (χ^2 trend). As birth weights and gestational ages did not appear to follow a normal distribution, the Mann–Whitney test was used to compare the missing eligible babies with those of the study population. The Kruskal—Wallis test was used to compare the birth weights and gestational ages of the study population in each of the three epochs. In all cases a p value <0.05 was taken to indicate statistical significance.

The Lothian Research Ethics Committee were contacted and it was declared that no ethical approval was required for this research.

RESULTS

Lothian population epidemiological data—supplied by ISD (table 1)

The proportion of babies born with birth weight <1500 g and/or gestational age <32 weeks who survived to 42 weeks' corrected gestational age (CGA) has increased from 1990 to 2004 ($p<0.001$ using χ^2 trend). This increase in survival is evident despite the proportion of these babies being born remaining unchanged ($p = 0.271$ using χ^2).

Study population—from Lothian hospitals data

During the study period, there were 1450 eligible babies registered for eye screening; 77 (5%) were discharged home prior to eye screening, or failed to attend outpatient eye screening. There were insufficient medical records from 10 (0.7%) babies. Thus complete data were available on 1363 infants (1363/1450, 94% of eligible population). ISD reported a total of 1636 babies from 1990 to 2004 with birth weight <1500 g and/or gestational age <32 weeks where the baby survived to CGA 42 weeks or more (table 1). Therefore, a discrepancy of 186 babies remains between our hospital data and ISD data. We could not access details on these babies due to

Table 1 Lothian population epidemiological data (supplied by ISD Scotland)

Time periods	Livebirths	Livebirths BW <1500 g and/or GA <32 weeks (% of livebirths)	Livebirths BW <1500 g and/or GA <32 weeks admitted to Lothian neonatal units	Livebirths BW <1500 g and/or GA <32 weeks surviving to CGA 42 weeks (% of livebirth BW <1500 g and/or GA <32 weeks)*
1990–4	47 937	701 (1.5)	637	586 (84)
1995–9	44 410	596 (1.3)	547	518 (87)
2000–4	40 833	587 (1.4)	564	532 (91)
Total	133 180	1884 (1.4)	1748	1636 (87)

*χ^2 trend shows evidence towards increased survival 1990–2004 ($p<0.001$).
BW, birth weight; CGA, corrected gestational age; GA, gestational age.

Table 2 Incidence of retinopathy of prematurity (ROP) in Lothian hospitals study population (1363 babies)

Time period	Total number of babies	Number of babies with any degree of ROP (% of total number)*	Number of babies with severe ROP (% of total number)†	Number of babies treated for ROP (% of total number)‡
1990–4	442	96 (22)	53 (12)	41 (9)
1995–9	447	94 (21)	39 (9)	21 (5)
2000–4	474	81 (17)	45 (9)	24 (5)

Severe ROP = stage 3 or greater ROP.
*No evidence of a statistically significant difference ($\chi^2 = 3.628$, $p = 0.16$; χ^2 trend = 3.13, $p = 0.08$).
†No evidence of a statistically significant difference ($\chi^2 = 2.872$, $p = 0.24$; χ^2 trend = 1.52, $p = 0.22$).
‡Evidence of a statistically significant difference ($\chi^2 = 9.789$, $p<0.01$; χ^2 trend = 6.686, $p<0.01$).

patient confidentiality and data protection legislation and therefore could not cross-check our hospital records with ISD.

The median gestational age for the Lothian hospitals study population (1363) was 29 weeks, (interquartile range 28–31), median birth weight was 1240 g (interquartile range 965–1490). The study population comprised 54% boys. The median gestational age of the missing eligible babies (enough data only from 77 discharged babies) was 31 weeks (interquartile range 30–32) and median birth weight was 1467 g (interquartile range 1340–1675). Using the Mann–Whitney test we found that the 77 babies had a significantly higher gestational age ($p<0.001$) and also a higher birth weight ($p<0.001$). These baseline characteristics were expected as only the more mature and heavier babies would have been discharged prior to commencement of eye screening.

The baseline characteristics of infants in the three time epochs, 1990–4, 1995–9 and 2000–4, were also calculated. There was no evidence of statistically significant differences in birth weight (Kruskal–Wallis, $p = 0.48$) or gestational age at birth (Kruskal–Wallis, $p = 0.09$) between the three cohorts.

Incidence and severity of ROP in Lothian hospitals study population (table 2)

One baby in 1999 developed stage 4 ROP after laser treatment. No babies developed stage 5 ROP during the study period. The heaviest baby treated weighed 1190 g at birth and the most mature baby treated was 30 weeks' gestation at birth.

Incidence and severity of ROP in Lothian hospitals study population for birth weight categories (tables 3 and 4)

From Lothian hospitals study population data, a total of 1032 babies with birth weight <1500 g underwent eye screening from 1990 to 2004. The remaining 331 babies had gestational ages <32 weeks but birth weights >1500 g and were not included in this analysis. A trend towards a greater proportion of babies with birth weight <750 g having no ROP ($p = 0.03$) was observed, and a reduced proportion of these babies required treatment ($p<0.01$). The incidence of severe ROP in babies with birth weight 1000–1249 g and 1250–1499 g was consistently low and there was a trend towards fewer 1250–1499 g birth weight babies having any ROP ($p = 0.04$). No statistically significant trends from 1990 to 2004 were observed for babies with birth weight 750–999 g and 1000–1249 g.

Incidence and severity of ROP in Lothian hospitals study population for gestational age categories

Similar trends were observed when considering the babies in gestational age categories (see data supplement online).

DISCUSSION

The changing trends in the incidence of ROP have been the cause of much debate. Improved survival rates for premature infants led to the concern that the incidence of ROP might increase.[6 24] Conversely, improved neonatal care led to a reported decrease in the incidence of ROP in some centres.[8 13–15]

Table 3 Incidence of retinopathy of prematurity (ROP) in Lothian hospitals study population with birth weight <750 g and 750–999 g

	Total babies screened in Lothian		Babies with no ROP (% of babies screened)		Babies with stages 1,2 ROP (% of babies screened)		Babies with stage 3 ROP, untreated (% of babies screened)		Babies with stage 3 ROP, treated (% of babies screened)	
Birth weight (g)	<750	750–999	<750*	750–999	<750	750–999	<750	750–999	<750†	750–999
1990–4	35	92	6 (17)	50 (54)	6 (17)	16 (17)	4 (11)	7 (8)	19 (54)	19 (21)
1995–9	50	61	9 (18)	37 (61)	15 (30)	15 (25)	11 (22)	4 (7)	15 (30)	5 (8)
2000–4	48	83	18 (38)	51 (61)	7 (15)	16 (19)	11 (23)	6 (7)	12 (25)	10 (12)

*Linear trend towards increased proportion of babies with no ROP ($\chi^2 = 6.489$, $p = 0.04$; χ^2 trend = 5.050, $p = 0.03$).
†Linear trend towards reduced proportion of babies treated ($\chi^2 = 8.418$, $p = 0.01$; χ^2 trend = 7.148, $p<0.01$).

Table 4 Incidence of retinopathy of prematurity (ROP) in Lothian hospitals study population with birth weight 1000–1249 g and 1250–1499 g

	Total babies screened in Lothian		Babies with no ROP (% of babies screened)		Babies with stages 1,2 ROP (% of babies screened)		Babies with stage 3 ROP-untreated (% of babies screened)		Babies with stage 3 ROP-treated (% of babies screened)	
Birth weight (g)	1000–1249	1250–1499	1000–1249	1250–1499*	1000–1249	1250–1499	1000–1249	1250–1499	1000–1249	1250–1499
1990–1994	97	101	81 (84)	95 (94)	13 (13)	5 (5)	0 (0)	1 (1)	3 (3)	0 (0)
1995–1999	104	126	83 (80)	121 (96)	17 (16)	5 (4)	3 (3)	0 (0)	1 (1)	0 (0)
2000–2004	118	117	106 (90)	116 (99)	7 (6)	0 (0)	3 (3)	1 (1)	2 (2)	0 (0)

*Linear trend towards increased proportion of babies with no ROP ($\chi^2 = 4.301$, $p = 0.12$; χ^2 trend = 4.224, $p = 0.04$).

Our prospective population based study has found a significant reduction in the number of babies treated for ROP from 1990 to 2004. A reduction in the overall incidence of any degree of ROP and severe ROP was also observed. We have also seen an increase in overall survival in babies with birth weight <1500 g or gestational age <32 weeks.

Our observed changes are based on a complete population rather than single hospital data and therefore referral and inclusion bias should be eliminated. Interobserver variation should be minimal for detection of severe ROP as only two examiners examined the population over the 15 year period and both were experienced paediatric ophthalmologists. The methods of ophthalmoscopic examination for ROP and the classification system used were the same for the whole study period. We have not, however, analysed our incidence of mild (stage 1 or 2) ROP, as these data are less robust due to likely inherent observer error in scoring these lesser grades. There may have been an under-reporting of "any degree of ROP" and a related over-reporting of "no ROP" as a consequence of failure to detect minor degrees of ROP. The context of this prospective data acquisition was as a clinical service rather than a precisely defined epidemiological project as in other studies.[17] We did not formally record ethnicity but results from the 2001 census in Scotland report the Scottish population as being 98% Caucasian, 1.4% Asian and 0.6% African Caribbean and other ethnic background.[25] There was minimal movement of babies between different centres. Oxygen saturation monitors were introduced in the late 1990s and the target limits have always been 86–94%. Arterial catheters were infrequently used throughout the study period with the target partial pressure of oxygen being 6–10 kPa.

Recent population studies report differing trends in the incidence of ROP. Chiang *et al* report a 20.3% incidence of any ROP in infants with birth weight <1500 g born in New York State between 1996 and 2000 which is a similar incidence to the present Lothian study.[20] Larsson *et al* compared ROP rates during 1988–90 and 1998–2000 in Sweden and found no change in incidence of any ROP and no change in severe ROP in infants with birth weight <1500 g.[26] Todd *et al* studied the incidence and treatment of severe ROP in New South Wales and the Australian Capital Territory from 1992 to 2002.[19] They found a considerable increase in severe ROP in infants ≤24 weeks' gestation (42% to 54%) together with an increase in treatment for severe ROP (19% to 24%). In infants of 25–26 weeks' gestation there was a marked decrease in severe ROP (26% to 19%) and there was no change in infants with gestational age 27–29 weeks. In the most recent population-based study to be carried out in the UK, Hameed *et al*[21] reported on severe ROP in Leicestershire from 1990 to 1999 in infants with birth weight ≤1250 g. They found an increase in severe ROP from 4% in 1990–4 to 12% in 1995–9. This increase in severe ROP was also observed in infants with birth weight <750 g. This is very different from our findings as we observed a reduction in the incidence of severe ROP in babies <750 g. The reason for the different outcomes is unknown but the incidence of severe ROP may be influenced by relatively minor changes in neonatal care policies and practice.

The papers discussed above are all population studies although most incidence papers published have observed the trends in either a single hospital or a group of hospitals. These findings can be difficult to relate to the population as a whole due to local regional differences in survival rates, neonatal management and ethnicity, but large hospital-based studies over long periods of time still provide useful information. Rowlands *et al* found a significant reduction in the incidence of severe ROP from 1989 to 1998 in a level 2 neonatal unit in London.[9] Hussain *et al* reported on a similar time period (1989–97) from a level 3 neonatal unit in the USA and found an incidence of 21% for any stage ROP and 5% for severe ROP.[8] These are very similar to our findings.

In the past decade, several publications have highlighted the role of oxygen free radicals in several neonatal disease processes, as well as ROP.[27–29] As a result, many neonatal units have implemented new oxygen saturation policies to reduce the amount of supplemental oxygen given to premature babies. Several large centres have reported a significant decrease in the incidence of severe ROP following introduction of lower oxygen saturation targets.[12 14 15] We did not change our oxygen saturation policy throughout the period of the study.

In summary, we have seen an increase in survival of preterm infants, a reduction in the incidence of any degree of ROP, severe ROP and a marked reduction in treatment for ROP in Lothian from 1990 to 2004. The past 20 years have seen dramatic advances in obstetric and neonatal care with routine administration of antenatal corticosteroids for premature labour, use of surfactant therapy, new methods of neonatal mechanical ventilation, introduction of continuous pulse oximetry, use of computerised monitoring systems and advances in neonatal nutritional support. It is highly probable that these advances are responsible for the overall decreasing incidence of ROP.

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Retinopathy of prematurity in small-for-gestational age infants compared with those of appropriate size for gestational age

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ABSTRACT

Aim: To compare the incidence of retinopathy of prematurity (ROP) in small-for-gestational age (SGA) infants compared with appropriate-for-gestational age (AGA) infants undergoing eye screening in the Lothian region of south east Scotland 1990–2004.

Methods: All infants in Lothian born with birth weight <1500 g and/or gestational age <32 weeks underwent eye screening by two experienced paediatric ophthalmologists. The presence of any stage of ROP (1–5), severe (stage 3 or greater) ROP and treated ROP was compared between the SGA and AGA infants using χ^2 or Fisher exact tests. SGA was defined as birth weight below the 10th centile for gestational age.

Results: A total of 1413 babies with birth weights <1500 g and/or gestational age <32 weeks underwent eye screening; 329 (23%) were SGA. SGA infants born at gestational ages 26–31 weeks were more likely to develop any stage of ROP ($p<0.01$) than their AGA peers. SGA infants were also more likely to develop severe ROP (gestational age 26–27 weeks, $p<0.01$; 28–29 weeks, $p = 0.01$; 30–31 weeks, $p = 0.01$).

Conclusions: SGA infants who underwent eye screening in the Lothian region of south east Scotland from 1990 to 2004 were significantly more likely to develop ROP and more severe disease than AGA infants.

What is already known on this topic

Small-for-gestational age (SGA) infants are at higher than average risk of morbidity and mortality from many neonatal disorders, including retinopathy of prematurity (ROP).

What this study adds

In this large cohort of 1413 infants, SGA infants born at 26–31 weeks' gestation were more likely to develop any stage of ROP or severe ROP.

Retinopathy of prematurity (ROP) is a vasoproliferative disorder of the eye affecting premature neonates. It is a major cause of childhood blindness in both the developed and developing worlds.[1] The pathogenesis of ROP is multifactorial, although the most recognised risk factors are low birth weight and low gestational age.[2] Small-for-gestational age (SGA) infants are a vulnerable population. They have a greater than average risk of morbidity and mortality from many neonatal disorders and have been reported to be at increased risk of developing ROP.[3–7] They are also at increased risk of developing cardiovascular disease and type 2 diabetes in later life.[8 9] Research into the mechanisms of perinatal growth is of major interest in order to try to minimise these risks and optimise the care of these infants. We report and compare the incidence of ROP in SGA and appropriate-for-gestational age (AGA) infants who underwent eye screening in the Lothian region of south east Scotland over a 15-year period from 1990 to 2004.

MATERIALS AND METHODS

Study population

In the Lothian region of south east Scotland, all infants born at gestational age (GA) <32 weeks and/or birth weight <1500 g have their eyes screened for ROP. GA was determined by early ultrasound examination. Infants were screened at one of three hospitals:

1. The Simpsons Memorial Maternity Pavilion/ The New Royal Infirmary, Edinburgh: level 3 south east Scotland Regional Neonatal Unit. Hospital moved to the site of the New Royal Infirmary, Edinburgh in summer 2002.
2. The Eastern General Hospital, Edinburgh: level 2 neonatal unit. Hospital closed in April 1998.
3. St Johns Hospital, Livingston: level 2 neonatal unit.

Eligible babies in these hospitals were referred to the ophthalmology team by nursing staff.

Eye examination schedule

Two dedicated paediatric ophthalmologists (BWF and EW) carried out all eye screening in Lothian. Infants were first examined at 4–6 weeks' chronological age, or 34 weeks' corrected age, whichever was earlier. Screening was repeated fortnightly until full retinal vascularisation. If "prethreshold" disease was found (see below for definition), screening was carried out weekly. Eye examinations continued after discharge from hospital if the vasculature was still immature when the infants left.

Eye examination technique

Pupils were dilated with topical 2.5% phenylephrine and 0.5% tropicamide. Drops were applied 60 min and 30 min before the eye examination. Indirect ophthalmoscopy was performed using a binocular indirect ophthalmoscope and 28 dioptre lens. A lid speculum and scleral indenter were

routinely used. Retinopathy was graded according to the international classification of ROP.[10] The zone of vascularisation, presence or absence of "plus" disease, number of clock hours of ROP, and the stage of ROP (1–5) were recorded. Plus disease represented significant dilatation and tortuosity of posterior pole blood vessels.[10] "Threshold" disease referred to five or more contiguous, or eight or more cumulative, clock hours of stage 3 ROP in zones 1 or 2 in the presence of plus disease.[11] "Prethreshold" ROP consisted of any stage 3 disease less extensive than threshold, or stage 2 disease in the presence of plus.[11] If threshold ROP was present, eyes were treated with cryotherapy in 1990–1991 and laser therapy from 1992 onwards.

Data collection

After every eye examination, the ophthalmologists recorded the date of birth, sex, GA, birth weight and maximum severity of ROP reached in any one eye for every baby examined. This information was stored on a database. The study included any baby born between 1 January 1990and 31 December 2004. The database was not analysed after 2004 because new treatment criteria were used from January 2005 following the publication of the ETROP study.[12] For every baby, the birth weight was plotted on a sex-specific growth chart.[13] An infant was defined as being SGA if their birth weight was below the 10th centile for GA.

Statistical analysis

For analysis, the maximum severity of ROP in either eye for an individual infant was recorded. Statistical analysis was performed using SPSS and the GraphPad Instat program. As birth weights and GAs did not follow a normal distribution, Mann–Whitney tests were used to compare the baseline characteristics of the SGA and AGA babies. Contingency tables were analysed in GA categories using χ^2 test with Yate's continuity correction. The Fisher exact test was used when numbers were small. In all cases, a two-sided p value of <0.05 was taken to indicate significance.

The Lothian research ethics committee declared that ethical approval was not required for this research.

RESULTS

Study population

In Lothian, from 1990 to 2004 there were 1748 live births with birth weight <1500 g and/or GA <32 weeks admitted to Lothian neonatal units.[14] Of these infants, 1668 were registered for eye screening. The difference between the number of live births and the number registered for screening was due to 80 babies dying before reaching screening age. Of the infants registered, 168 (10%) were transferred to a neonatal unit outwith Lothian before eye screening, 77 (5%) were discharged home before eye screening or failed to attend outpatient eye screening, and there were insufficient medical records for 10 (<1%). Thus complete data were available for 1413 infants. Just under a quarter (329/1413; 23%) of the study population were SGA. Table 1 shows the baseline characteristics.

The SGA infants had a higher median birth weight and GA than the AGA infants (Mann–Whitney $p<0.0001$ for both). There were more female infants than male infants in the SGA cohort.

Prevalence and severity of ROP

Table 2 presents eye findings. One AGA baby in 1999 developed stage 4 ROP after laser treatment. No babies developed stage 5 ROP during the study period. The numbers of AGA and SGA infants with any stage of ROP (1–5), severe (stage 3 or greater) ROP and treated ROP were compared using the χ^2 test, or the Fisher exact test when cell numbers were small. Table 2 gives the findings. SGA infants born at GA 26–31 weeks were more likely to develop any stage of ROP ($p<0.01$) than their AGA peers. They were also more likely to develop severe ROP (GA 26–27 weeks, $p<0.01$; GA 28–29 weeks, $p = 0.01$; GA 30–31 weeks, $p = 0.01$).

DISCUSSION

Our ROP eye screening data gathered over 15 years show that SGA infants (GA 26–31 weeks) are more likely to develop any stage of ROP and severe ROP than their AGA peers. We did not find this association to be significant in infants of GA ≤25 weeks. This may have been because of the small numbers at this gestation, but also because these infants are at a high risk of ROP irrespective of whether they are SGA or AGA. We also did not find a significant difference in SGA and AGA infants of GA >32 weeks and this is almost certainly due to the low incidence and low risk of developing ROP at this GA. When the numbers of SGA and AGA infants treated for ROP were compared with the numbers of those with ROP but not treated, SGA infants were no more likely to require laser treatment for severe ROP. Again, this finding can probably be explained by the small numbers with treated ROP.

Our study is useful, as our findings are based on a large cohort of babies over a long period of time with two consistent observers and methodical documentation of eye examinations. The two specialised ophthalmologists (EW and BF) have worked together using indirect ophthalmoscopy to perform all the ROP eye screening examinations in the Lothian region since 1990. In 2007, for the purposes of another study, the examiners undertook interobserver agreement studies by independently and blindly grading 80 clinical ROP screening Retcam images. They showed 95% agreement for the presence or absence of plus disease, 94% agreement for stage of ROP, and 97% agreement for zone of ROP. We are therefore confident that interobserver variation was minimal. The methods of ophthalmoscopic examination for ROP and the classification system used were the same over the whole study period. Ethnicity was not formally recorded, but the 2001 census in Scotland reported the population as being 98% Caucasian, 1.4% Asian and 0.6% Afro-Caribbean and other ethnic background.[15] As both level 2 and 3

Table 1 Baseline characteristics of the study population

	Number (male:female)	Birth weight (g)*	GA (weeks)*	Number of GA ≥32 weeks
AGA infants	1084 (617:438)†	890 (585–1170)	27 (26–31)	25
SGA infants	329 (130:194)‡	1035 (825–1235)	31 (29–32)	125

*Median (interquartile range).
†Sex not recorded for 29 babies.
‡Sex not recorded for five babies.
AGA, appropriate-for-gestational age; GA, gestational age; SGA, small-for-gestational age.

Table 2 Maximum severity of retinopathy of prematurity (ROP) in appropriate-for-gestational age (AGA) and small-for-gestational age (SGA) babies (and the significance of differences)

	≤25 weeks	26–27 weeks	28–29 weeks	30–31 weeks	≥32 weeks
Without ROP (AGA:SGA)	27:0	112:6	265:51	429:83	25:123
Stages 1,2 ROP (AGA:SGA)	31:3	39:10	25:10	15:11	0:1
Stage 3 ROP untreated (AGA:SGA)	19:0	12:11	4:5	0:1	0:1
Stage 3 ROP treated (AGA:SGA)	51:3	28:8	2:1	0:1	0:0
p Value of difference*					
Any stage of ROP	0.46	**<0.01**	**<0.01**	**<0.01**	1.00†
Severe (stage 3–5) ROP	1.00†	**<0.01**	**0.01**	**0.03**†	1.00†
Treated ROP	1.00†	0.59	1.00†	0.46†	–

*p Values relate to comparison of numbers of AGA and SGA infants using χ^2 test; p values not corrected for the multiple comparisons. p Values in bold indicate significant difference.
†Fisher exact test used because of small numbers.

units were involved, there may have been some variations in neonatal care. However, there were no changes in our target oxygen saturation policies during the study period.

SGA infants are a vulnerable population at higher risk of perinatal morbidity and mortality than their appropriately sized peers.[3–6] Other studies have also reported on the incidence of ROP in SGA and AGA infants with similar findings to ours.[4 6 7] Gortner *et al*[6] reported the incidence of ROP in SGA infants to be more than double that in AGA infants (37% vs 15%), although there was no difference in prevalence of stage 3 disease between the two groups. Bardin *et al*[4] compared two historical cohorts of SGA and AGA infants born at 24–26 weeks' gestation. Like us, they found SGA infants to be at increased risk of developing any stage of ROP (90% vs 58%) and severe ROP (65% vs 12%). Allegaert *et al*[7] showed that SGA infants were 3.7 times more likely to develop threshold ROP than their AGA peers. They went on to document perinatal growth and found that, even when growth-restricted (birth weight <25th centile) infants displayed normal postnatal growth (ie, the same g/kg/day as AGA infants), they still had a higher risk of developing threshold ROP.[7] Experiments in rat models of ROP also support these clinical findings. Rats raised in expanded litters undergo postnatal growth restriction. These growth-restricted rats have been found to have more abnormal retinal neovascularisation in two well-established models of ROP.[16 17]

There are many possible explanations for why SGA infants have an increased incidence of ROP. Most SGA infants are small as a result of intrauterine growth restriction. Such infants are exposed to changes in organ development because of fetal hypoxaemia, nutrient restriction and an altered endocrine environment.[18] These developmental changes may be linked to the increase in ROP. SGA infants are often sicker than their AGA peers, requiring more intensive and more prolonged hospital care.[19] They are therefore more likely to require supplemental oxygen, which is a well-documented risk factor for ROP.[20] SGA infants have lower serum concentrations of insulin-like growth factor 1, and there is evidence for a role for this growth factor and vascular endothelial growth factor in the pathogenesis of ROP.[21]

In conclusion, SGA infants born in Lothian from 1990 to 2004 were significantly more likely to develop any stage of ROP and more severe ROP than AGA infants.

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Wide-field digital retinal imaging versus binocular indirect ophthalmoscopy for retinopathy of prematurity screening: a two-observer prospective, randomised comparison

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ABSTRACT

Aim: To compare the diagnostic accuracy of wide-field digital retinal imaging (WFDRI) with the current "gold standard" of binocular indirect ophthalmoscopy (BIO) for retinopathy of prematurity (ROP) screening examinations.

Methods: A consecutive series of premature infants undergoing ROP screening at Edinburgh Royal Infirmary were eligible for recruitment into this prospective, randomised, comparative study. Infants were screened using both WFDRI (Retcam II with neonatal lens) and BIO by two paediatric ophthalmologists who were randomised to the examination technique. Both examiners documented their clinical findings and management plans in a masked fashion. WFDRI eye findings were compared with those of BIO.

Results: A total of 81 infants were recruited, and information from 245 eye examinations was analysed. The sensitivity of WFDRI in detecting any stage of ROP, stage 3 ROP and "plus" disease was 60%, 57% and 80%, respectively, and specificity 91%, 98% and 98%, respectively. The proportional agreement between WFDRI and BIO was 0.96 for detecting stage 3 disease and 0.97 for detecting "plus" disease. There was very good agreement on management decisions (kappa 0.85).

Conclusion: When used in a routine ROP screening setting, a randomised comparison of WFDRI and BIO, WFDRI showed relatively poor sensitivity in detecting mild forms of ROP in the retinal periphery. This resulted in difficulty in making decisions to discharge infants from the screening programme. Sensitivity was better for more severe forms of ROP, but at present WFDRI should be regarded as an adjunct to, rather than a replacement for, BIO in routine ROP screening.

Retinopathy of prematurity (ROP) is a vasoproliferative disorder of the eye affecting premature infants and is an important cause of childhood blindness.[1] Early detection and treatment of Type 1 ROP as defined by the Early Treatment of ROP (ETROP) study has been shown to significantly reduce the incidence of severe visual loss.[2]

Screening guidelines have been developed in many countries with the aim of identifying premature infants with potentially sight-threatening disease. The conventional gold standard screening technique is binocular indirect ophthalmoscopy (BIO) with scleral indentation. Current screening for ROP by this technique is difficult, as it requires a specialised paediatric ophthalmologist, and adequate ophthalmic expertise is often confined to larger regional centres. Provision of satisfactory ophthalmology services for these babies is challenging in developed countries,[3] and even more difficult in "middle income" countries.[1]

A wide-field digital camera system (Retcam II, Clarity Medical Systems, Pleasanton, California) has been developed that can image the retina of premature infants.[4–9] Store and forward telemedicine using this method potentially enables local ophthalmologists, neonatologists or neonatal nurses to capture images at a remote location for transmission and interpretation by a regional or international expert.[5–7 9–14]

A number of studies have compared the performance of wide-field digital retinal imaging (WFDRI) and binocular indirect ophthalmoscopy (BIO) in ROP screening.[5 6 8 9 14–18] All these studies have a similar methodology whereby the same or different examiners perform WFDRI and BIO imaging on an infant. The stored WFDRI images are then cleared of any patient identifiable data and interpreted at a later date by either the study examiner or an independent expert.

We have carried out a masked, double-observer prospective randomised comparison of WFDRI and BIO in a consecutive series of ROP screening examinations in one nursery. We used two expert examiners who were randomised to WFDRI or BIO examination technique and remained masked to each other's findings throughout the study. Clinical findings and management decisions were made at the time of clinical examination.

METHODS

This was a prospective, randomised, comparative study. The Local Research Ethics Committee (LREC) reviewed and approved the study. All parents gave informed consent.

Study population

Consecutive infants undergoing routine ROP screening at Edinburgh Royal Infirmary Neonatal Intensive Care Unit were eligible for inclusion in the study. At this centre, all babies born with gestational age less than 32 weeks or birth weight less than 1500 g have their eyes screened for ROP. Patients were recruited from June 2004 to May 2007.

Examination schedule

Screening was carried out by two experienced paediatric ophthalmologists (BWF and EW). Infants were first examined at a chronological age

of 4–6 weeks or corrected age of 34 weeks, whichever was earlier. Screening was continued fortnightly if no ROP was present and weekly if any ROP was seen. Screening continued until the retina was normally vascularised into zone 3, the infant required treatment or until the infant was transferred to an outlying hospital. On each screening occasion, study infants had both eyes examined by both WFDRI and BIO. The study coordinator (CD) randomised both examiners to screening using either WFDRI or BIO and also randomised the order in which the examinations were to be carried out. Randomisation was carried out for each examination, using sealed envelopes.

Examination technique

Pupils were dilated with topical phenylephrine 2.5% and tropicamide 0.5% applied 60 min and 30 min prior to the eye examination. One drop of oxybuprocaine 0.4% was applied to each eye immediately prior to examination. BIO was performed using a 28 dioptre lens. A lid speculum and scleral indenter were used. WFDRI images were recorded with the Retcam II Digital Retinal Camera (Clarity Medical Systems) with the neonatal nose-cone. A lid speculum was used, and polyacrylic acid gel was applied to the anaesthetised cornea before capturing the images. The study coordinator timed how long each eye examination took from the insertion of the eyelid speculum to its removal at the end of the examination.

All examinations were performed with cardiac and oxygen saturation monitoring. The examinations were interrupted if an infant's heart rate or oxygen saturation decreased to an unacceptably low level and recommenced once these had stabilised.

Clinical findings

Both examiners graded retinopathy according to The International Classification of ROP.[19] The stage of ROP, zone of vascularisation, number of clock hours of ROP and presence or absence of "plus" disease were documented. "Plus" disease represented significant dilatation and tortuosity of posterior pole blood vessels meeting or exceeding that of a standard photograph.[19] "Threshold" ROP, as defined in the CRYO-ROP trial, referred to stage 3 ROP in zone 1 or 2, of five or more contiguous or eight or more cumulative clock hours in the presence of "plus" disease.[20] All eyes with "threshold" ROP were treated with diode laser therapy from June 2004 to December 2004. From January 2005, new treatment criteria were used, following the publication of the ETROP study. Eyes with type 1 ROP were treated.[2] Type 1 ROP was defined as any stage of ROP with plus disease in zone 1; stage 3 ROP without plus disease in zone 1; or stage 2 or 3 ROP with plus disease in zone 2.[2] Both eyes were treated, even when one eye did not fully meet treatment criteria.

Table 1 Diagnostic comparison of the two examiners

	Examiner 1 BIO	Examiner 2 BIO	Examiner 1 WFDRI	Examiner 2 WFDRI
Plus disease (eyes)	5	5	7	4
Stage 3 ROP (eyes)	7	7	6	7
Any ROP (eyes)	26	19	25	21
Treat (infants)	3	3	4	3
Discharge (infants)	35	34	31	31

BIO, binocular indirect ophthalmoscopy; ROP, retinopathy of prematurity; WFDRI, wide-field digital retinal imaging.

Examination documentation and management plans

Each examiner documented their findings in separate books and remained masked to the other examiner's findings, and to their own findings from previous weeks. Based on their findings, each examiner documented a management plan, which was to discharge the infant, treat the infant or examine the infant again in either 1 or 2 weeks' time. The study coordinator (CD) read each examiner's management plans. If there was any difference in management plan then the infant was seen again the following week. This continued until both management plans were the same. If a decision to treat was reached using BIO but not using WFDRI then the infant was seen the following week. If the infant still warranted treatment the following week as judged by BIO then the infant was treated even if a discrepancy remained with the WFDRI management plan. The study coordinator was present at all examinations to ensure that examiners remained masked to each other's findings.

Standardisation of examiners

The two specialised paediatric ophthalmologists (BF and EW) have worked together using BIO to perform all ROP eye screening examinations in the Lothian region since 1990. In order to measure interobserver agreement, the examiners independently analysed Retcam images from 81 clinical ROP screening examinations from the study. The stage of ROP seen, the presence or absence of plus disease and the location of the disease were documented.

Data interpretation and statistical method

For each eye examined by BIO and WFDRI, the highest stage of ROP, the presence or absence of plus disease and the management plan were recorded and used for analysis. The WFDRI findings were compared with those of the current "gold standard" of BIO, and the sensitivity and specificity, together with 95% confidence intervals, were calculated. The WFDRI management plan reached for each infant after both eyes were examined was compared with the plan from the "gold standard" BIO by calculating the kappa value as a marker of technique agreement.

RESULTS

Patients and study examinations

A total of 81 infants were recruited from May 2004 to June 2007. The median gestational age at birth of study infants was 29 weeks (interquartile range 27 to 31), and a median birth weight was 1225 g (interquartile range 878 to 1492). A total of 123 eye examinations on both eyes were carried out (246 eyes examined in total). On one occasion it was not possible to obtain adequate quality images from one eye using WFDRI. Findings from 245 eyes were therefore analysed. Fifty-four infants contributed one examination, 20 infants contributed two examinations, three infants contributed three examinations, two infants contributed four examinations, one infant contributed five examinations, and one infant contributed

Table 2 Detection of any stage of retinopathy of prematurity (ROP)

	BIO ROP present	BIO no ROP
WFDRI ROP present	27	19
WFDRI no ROP	18	181

Sensitivity = 60% (44–74%). Specificity = 91% (86–94%).
BIO, binocular indirect ophthalmoscopy; ROP, retinopathy of prematurity; WFDRI, wide-field digital retinal imaging.

Table 3 Detection of stage 3 disease

	BIO stage 3 present	BIO no stage 3 disease
WFDRI stage 3 present	8	5
WFDRI no stage 3 disease	6	226

Sensitivity = 57% (29–82%). Specificity = 98% (95–99%).
BIO, binocular indirect ophthalmoscopy; WFDRI, wide-field digital retinal imaging.

seven examinations on both eyes. Not every examination of every infant was included, as study examinations could not be performed when one examiner was unavailable. Almost all examinations were performed at postconceptual age 32–36 weeks. In this cohort, BIO detected 24 infants who developed ROP (24/81, 30%) and five infants (5/81, 6%) that required treatment. On 63/123 examinations, BWF used WFDRI, and EW used BIO.

Standardisation of examiners

The two examiners demonstrated 95% agreement (95% confidence intervals 91 to 98) on the presence or absence of plus disease, 94% agreement on the stage of ROP (95% confidence intervals 91 to 98) and 97% agreement (95% confidence intervals 95 to 99) on the zone of ROP following the independent, masked scoring of 81 Retcam examinations. In addition, the study data were analysed for systematic bias between the two examiners in each diagnostic category, for BIO and for WFDRI. There were no significant systematic differences in any diagnostic category, using the Fisher exact test (table 1).

Detection of disease

The detection of any stage of ROP, stage 3 disease and plus disease is shown in tables 2–4.

Proportional agreement

The proportional agreement of the two examination methods is shown in table 5.

Patient management decisions

Following the eye examination, each examiner decided a management plan for the infant choosing one out of three possible outcomes—treat the infant, discharge the infant or review the infant in 1 or 2 weeks' time. The kappa value for agreement on management decisions between WFDRI and BIO was 0.85, which is very good agreement. Details of decisions to treat are given in table 6, and to discharge in table 7.

On two occasions the WFDRI examination decision was to treat the infant while the BIO examination decision was not to treat. One infant was re-examined the following week, and both WFDRI and BIO agreed that treatment was required. The second infant was re-examined the following week using BIO only (one examiner was absent) and was not treated.

On one occasion the BIO decision was to treat the infant, while the WFDRI decision was not to treat. The documented clinical findings for this individual case showed that the infant was borderline for treatment. When this case was re-examined 1 week later both BIO examination and WFDRI examination findings agreed that treatment was required.

On five occasions the WFDRI decision was to discharge the infant, and the BIO was to perform a further examination. On re-examination the following week there was agreement between the WFDRI and BIO in two cases. The remaining three infants had left the study due to transfer back to local hospitals, and detailed retinal examination findings were unknown. However, it was known that no infant subsequently required treatment. On 14 occasions, the BIO decision was to discharge the infant and the WFDRI not to discharge. There was agreement on management plan within 1 week in seven cases and 2 weeks in one case, and we do not have information on five infants who left the study due to transfer back to local hospitals. It was known that none of these infants subsequently required treatment.

Table 4 Detection of "plus" disease

	BIO plus present	BIO no plus
WFDRI plus present	8	5
WFDRI no plus	2	230

Sensitivity = 80% (44–97%). Specificity = 98% (95–99%).
BIO, binocular indirect ophthalmoscopy; WFDRI, wide-field digital retinal imaging.

Time taken to complete an examination

The median time taken for WFDRI examination of both eyes was 110 s (interquartile range 80 to 133). The median time taken for BIO examination of both eyes was 90 s (interquartile range 65 to 120). Examinations using BIO were significantly quicker (Mann–Whitney $p = 0.005$).

DISCUSSION

We compared the ability of WFDRI to detect ROP and guide patient management decisions compared with the current gold standard of BIO. We found the sensitivity of WFDRI in detecting any ROP, stage 3 ROP and plus disease to be 60%, 57% and 80%, respectively, with a specificity of 91%, 98% and 98%, respectively. There was excellent proportional agreement between the two screening methods for detecting stage 3 ROP and plus disease (0.96 and 0.97) and very good agreement on management decisions (kappa value 0.85).

A number of previous studies have compared the performance of WFDRI and BIO in ROP screening.[4–6 8 9 14 16] Roth *et al* found 82% sensitivity and 94% specificity of WFDRI compared with BIO for detecting any stage of ROP.[8] Shah *et al* found 86% sensitivity and 92% specificity of WFDRI compared with BIO in detecting any stage of ROP, and no case of threshold ROP was missed with WFDRI.[16] Yen *et al* used WFDRI to attempt to predict which eyes would develop threshold disease by evaluating WFDRI images at two distinct gestational time points. Low sensitivities and high specificities were recorded for detecting various stages of ROP.[14] The authors of these three studies thought their lower sensitivities were due in part to the technical difficulties encountered using a standard child lens attachment for the screening examinations. Visualisation of the peripheral retina was particularly poor. This technical limitation of camera size has now been partly overcome, and a specialised smaller neonatal nose cone has been developed, which we used in our study. However, we found difficulty in visualising the peripheral retina with this lens.

Table 5 Proportional agreement of the two examination methods

Outcome	Proportional agreement
Detection of any stage of retinopathy of prematurity	0.85
Detection of stage 3 disease	0.96
Detection of "plus" disease	0.97

The proportional agreement is represented by the number of times that BIO and WFDRI concur divided by the total number of examinations.

Table 6 Decision to treat (based on examination of a baby: 123 examinations in total)

	BIO treated	BIO not treated
WFDRI treat	5	2
WFDRI not treat	1	115

BIO, binocular indirect ophthalmoscopy; WFDRI, wide-field digital retinal imaging.

Table 7 Decision to discharge (based on examination of a baby: 123 examinations in total)

	BIO discharge	BIO not discharge
WFDRI discharge	56	5
WFDRI not discharge	14	48

The term "not discharge" refers to treat, or follow up.
BIO, binocular indirect ophthalmoscopy; WFDRI, wide-field digital retinal imaging.

We found that the peripheral retina could be visualised much more easily with the BIO than with WFDRI. As we studied a consecutive series of infants in our nursery, a relatively high proportion of our cases had mild or no ROP. Our series therefore included a higher proportion of cases that were relatively more difficult to diagnose with WFDRI, and this may partly explain the relatively low sensitivity results for any stage of ROP in our study.

Our results of low sensitivity for stage 3 disease and plus disease, normally visualised in the more posterior part of the retina, were lower than those reported in a number of other studies.[5 8 9 13 15–18] Ells *et al* reported a sensitivity of 100% and specificity of 96% in the detection of "referral warranted" ROP (any zone 1 disease, or any plus disease or stage 3 disease).[6] Chiang *et al* reported 100% sensitivity for the detection of ROP that required treatment.[5] The recently reported SUNDROP study found a sensitivity of 100% and specificity of 99% for the detection of ROP that required treatment.[17] Similarly, the multicentre Photo-ROP study reported a sensitivity of 92% and specificity of 37% for the detection of "clinically significant" ROP, and a sensitivity of 92% and specificity of 67% for the detection of ETROP study Type 1 ROP.[15]

In our study, all cases judged to require treatment following BIO examination were identified with WFDRI examination. One infant, with borderline findings, was thought to require treatment following indirect ophthalmoscopy examination but not following WFDRI examination. However, 1 week later a decision to treat was made by both observers. In our study the sensitivity of WFDRI examination in making treatment decisions was therefore 100%, allowing for a repeat examination after 1 week. Interestingly, the WFDRI examination management decision on two occasions was to treat the infant a week before BIO examination resulted in a decision to treat. Ells *et al* found that severe ROP was diagnosed by WFDRI at least 1 week before BIO in 43% of eye examinations.[6]

We found that the peripheral retina could be visualised much more easily with BIO than WFDRI. This resulted in a higher rate of decisions to discontinue screening examinations when using BIO, as visualisation of normal vascularisation into Zone 3 was much more readily achieved. Our study suggests that BIO is superior to WFDRI examination in making decisions to discharge infants from ROP screening. In nurseries where both methods are available, it may be more efficient to perform the anticipated final "discharge" examination of an infant using the BIO, even when the WFDRI is used for earlier examinations. In nurseries where BIO screening is not available, uncertainty about the end point of screening with the WFDRI requires a more prolonged screening programme for each infant.

The outcome of the ETROP study has shifted the emphasis of diagnostic decisions about treatment towards the detection of dilatation and tortuosity of the posterior retinal blood vessels (plus disease).[2] The emphasis in ROP screening has therefore moved to examination on the posterior retinal blood vessels. WFDRI is particularly effective in imaging the posterior retina. We found a 80% sensitivity and 98% specificity of WFDRI examination compared with BIO examination in the detection of plus disease. Other studies have found a very high sensitivity of WFDRI examination compared with BIO examination in the detection of plus disease.[6 15 18]

The interobserver agreement for the presence or absence of plus disease was high in our study, with 98% agreement. However, few cases with plus disease were included, and this figure may therefore be unreliably high. The interobserver agreement on the presence or absence of plus disease has been surprisingly low in some studies.[21–23] This may partly explain the variation in reported rates of treatment-requiring ROP in different hospitals.[24] The standard photograph originally used to define plus disease was a single, narrow-field photograph.[19] The updated classification of ROP[25] includes a wide-field standard photograph. A number of representative wide-field photographs, which have a severity of plus disease equivalent to the current standard narrow field photograph, would be helpful in educating users of wide-field images in the diagnosis of plus disease. The development of image analysis software that can quantify features of plus disease may remove subjectivity from this important area of decision-making.[26]

We have carried out a masked, double-observer prospective randomised comparison to determine the sensitivity and specificity of WFDRI in making diagnostic decisions in the nursery, compared with the current gold standard of BIO. The WFDRI showed relatively poor sensitivity in detecting mild forms of ROP. In nurseries where both methods are available, it may be more efficient to perform the anticipated final "discharge" examination of a infant using the BIO. Sensitivity was better for more severe forms of ROP, and no infants who required treatment were missed by WFDRI examination. However, at present WFDRI should be regarded as an adjunct to, rather than a replacement for, BIO in routine ROP screening.

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Pain in neonates during screening for retinopathy of prematurity using binocular indirect ophthalmoscopy and wide-field digital retinal imaging: a randomised comparison

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ABSTRACT

Objective To compare the pain experienced by premature infants undergoing wide-field digital retinal imaging (WFDRI) and binocular indirect ophthalmoscopy (BIO) for retinopathy of prematurity (ROP) screening.

Methods Infants were recruited at Edinburgh Royal Infirmary Neonatal Unit, Edinburgh, UK. Eyes were examined by WFDRI and BIO with eyelid speculum by two experienced paediatric ophthalmologists in random order. A pain score (Premature Infant Pain Profile (PIPP)) for WFDRI and BIO was generated.

Results A total of 76 infants were recruited. The (mean, SD) PIPP score for WFDRI was 15.0, 2.1 and for BIO was 15.2, 2.4 (paired t test p=0.47). The authors observed that infants started crying with corresponding physiological changes as soon as the eyelid speculum was inserted and crying stopped on speculum removal.

Conclusion WFDRI and BIO with eyelid speculum are similarly painful for infants. The authors speculate that the eyelid speculum rather than the examination method may contribute most to the pain experienced.

What is already known on this topic

- Wide-field digital retinal imaging and binocular indirect ophthalmoscopy are both used for routine retinopathy of prematurity eye screening.
- We have limited information about the pain experienced by neonates undergoing both techniques.

What this study adds

- Both screening methods are painful for infants but the pain experienced is of a similar severity for both techniques.
- We propose that the eyelid speculum, rather than the examination method contributes most to the pain experienced.

INTRODUCTION

Premature infants need regular screening for the early detection of retinopathy of prematurity (ROP) and the successful prevention of its blinding end stage.[1] The mainstay of screening is binocular indirect ophthalmoscopy (BIO), with eyelid speculum and scleral indentation. This technique must be carried out by an expert ophthalmologist. It is recognised as being painful, and analgesia should be used routinely for the procedure.[1]

The use of the digital retinal camera for wide-field digital retinal imaging (WFDRI) has been suggested as a replacement for BIO. WFDRI can be carried out by less specialist staff with the help of experts for interpretation using telemedicine. It has been suggested that WFDRI may be less painful than BIO.[2] We recently carried out a masked, double-observer prospective randomised comparison of the diagnostic accuracy of WFDRI with BIO.[3] We designed, as part of our investigation, a study to compare the pain effects of BIO and WFDRI screening processes and this report describes the findings.

METHODS

This was a prospective, randomised, comparative study. Ethical approval was granted by the Lothian Research Ethics Committee, and all parents gave written consent.

Subjects and study design

All infants recruited required routine ROP screening at Edinburgh Royal Infirmary Neonatal Intensive Care Unit between June 2004 and May 2007. Infants were excluded if they were requiring mechanical ventilation or analgesic medication or if they had moderate/severe neurological impairment (grade 3/4 intraventricular haemorrhage or periventricular leukomalacia) as it was felt that these infants would respond differently to painful stimuli. Infants were examined by two experienced paediatric ophthalmologists (BF and EW). Only the first screening examination for each baby was included in this study. Infants had both eyes examined by WFDRI and BIO. The study coordinator (CD) randomised (via sealed brown envelopes) both examiners to screening using either WFDRI or BIO and also randomised the order in which the examinations were to be carried out.

Eye examinations

Infants had their pupils dilated with topical phenylephrine 2.5% and tropicamide 0.5% applied 60 min and 30 min prior to eye examination.

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Infants were then handled minimally and were not exposed to painful procedures unless medically indicated. One drop of oxybuprocaine 0.4% was applied to each eye immediately prior to each eye examination. Infants were examined on a cot blanket. They were unswaddled and non-nested. The study coordinator held the infants arms gently across their chests and steadied their heads during both examinations. Pacifiers were not used and oral sucrose was not given. A lid speculum (Barraquer Oosterhuis child speculum) and scleral indenter (Schokett-style paediatric scleral depressor) was routinely used during BIO. WFDRI images were taken using the digital retinal camera (RetCam II; Clarity Medical Systems, Pleasanton, California, USA) with the neonatal nose cone. The same type of lid speculum was used but scleral indentation was not routinely performed. After the first examination by the first examiner, the infant was handled minimally and allowed to recover for at least 30 min, to allow physiological variables to return to baseline prior to undergoing the second eye examination. All infants had constant cardiac and oxygen saturation monitoring.

Pain monitoring and data interpretation

The Premature Infant Pain Profile (PIPP) scoring system was used.[4] Baseline observations were recorded and during the first minute of BIO and WFDRI, the maximum and minimum heart rate (HR) and minimum oxygen saturation of the infants were documented. The facial features of every infant were video recorded and videotapes were later reviewed by an independent observer (KD) who scored the facial features during the first minute of BIO and WFDRI according to the PIPP. KD could not be blinded to the type of examination. The PIPP scores, heart rates and oxygen saturations for WFDRI and BIO were compared using paired t tests generated from the GraphPad InStat program (GraphPad, La Jolla, California, USA). Fisher's exact test was used to compare the occurrence of marked bradycardias and desaturations. A p value <0.05 was taken to indicate statistical significance.

RESULTS

A total of 81 infants were recruited to our WFDRI/BIO diagnostic comparative trial.[3] Inadequate quality video recordings were obtained for four babies and one baby was excluded as he had a periventricular leukomalacia. Therefore, a total of 76 infants were included in this study. In all, 39 infants received BIO first, and 37 WFDRI first. The baseline characteristics of the study population are shown in table 1. There were 40 boy infants and 36 girls. A total of 50 babies were breathing room air, 13 babies had nasal prong oxygen and 13 babies were having nasal continuous positive airway pressure (CPAP) with oxygen.

Table 2 shows details of the infants' heart rates and oxygen saturation levels before and during both eye examinations. There were no differences in baseline levels between the two groups. The mean PIPP score for WFDRI was 15.0 (SD 2.1) and the mean PIPP score for BIO was 15.2 (SD 2.4). There was no statistically significant difference in PIPP scores (paired t test $p=0.47$).

We observed a significantly greater increase in HR during WFDRI than during BIO ($p=0.03$). There was no statistically significant difference in occurrence of marked bradycardias ((HR <100 bpm); Fisher's exact test $p=1.00$) or marked desaturations ((min O_2 sat <80%); Fisher's exact test $p=0.52$) during the two examination techniques.

Table 1 Baseline characteristics of the study population

	Mean (range)
Gestational age, weeks	28.6 (24–35)
Corrected gestational age at retinopathy of prematurity examination, weeks	34.1 (30–40)
Birth weight, g	1208 (610–1970)
Number of days intubated and ventilated	4.6 (0–45)
Number of days of morphine	0.2 (0–5)

Table 2 Heart rate and oxygen saturation details

	BIO, mean (SD)	WFDRI, mean (SD)
Baseline heart rate, bpm	148 (13)	147 (14)
Minimum heart rate during examination	134 (25)	133 (28)
Maximum heart rate during examination	168 (18)*	172 (17)*
Baseline oxygen saturation, %	96 (4)	96 (4)
Minimum oxygen saturation during examination	87 (12)	88 (10)

*Statistically significant difference comparing BIO and WFDRI maximum heart rates (paired t test $p=0.03$).
BIO, binocular indirect ophthalmoscopy; WFDRI, wide-field digital retinal imaging.

DISCUSSION

This study, where infants received BIO and WFDRI but in a random order, demonstrates that ROP eye screening is painful, but the pain experienced is similar for both techniques.

The PIPP was chosen as it is a well validated method for measuring procedural pain in preterm infants and uses contextual indicators (gestational age and behavioural state), physiological indicators (heart rate and oxygen saturation) and behavioural indicators (brow bulge, eye squeeze and nasolabial furrow) in its calculation.[4] The major drawback of using the PIPP score in this setting is that it only takes into account an increase in heart rate, and bradycardia during eye screening can occur due to the oculocardiac reflex. We therefore additionally recorded the minimum heart rates during all examinations but there was no significant difference in these between the two screening methods.

While carrying out this study we observed that infants immediately started crying with corresponding physiological changes as soon as the eyelid speculum was inserted and crying stopped on speculum removal. As WFDRI and BIO both require speculum use to obtain optimal retinal views, we propose that the speculum, rather than the examination method, may contribute most to the pain experienced. Other groups have reported marked physiological responses associated with speculum usage.[5] Perhaps more investigation should be directed to examination techniques that do not require the use of a speculum, or to improved speculum design and insertion technique. We are currently investigating the use of a small, relatively malleable Barraquer style single use neonatal speculum that can be slightly bent prior to use in order to set the blades closer together or wider apart in order to accommodate differing palpebral aperture sizes (Malosa Medical 7.00 mm closed blade, 0.8 mm wire; Malosa, Sowerby Bridge, UK).

There is currently much debate about whether WFDRI can be introduced to replace BIO screening examinations. Premature neonates are exposed daily to many painful stimuli and we have shown that WFDRI and BIO with eyelid speculum are similarly painful. Further work is required to implement adequate analgesia regimes for ROP screening.

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